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High affinity receptors for *Helicobacter pylori* and use thereof

FIELD OF THE INVENTION

5 The present invention describes novel high affinity receptors for *Helicobacter pylori*. The receptors comprise sialylated poly-N-acetyllactosamine structures. The present invention is directed to therapeutic and prophylactic uses of the substances against the major gastric pathogen *Helicobacter pylori*. Furthermore the present invention is directed to analytic and diagnostic uses of the structures. The present invention is
10 further directed to the uses of the structures in functional foods preventing *H. pylori* infections.

BACKGROUND OF THE INVENTION

15 Sialyllactose and sialic acid on glycoproteins or glycolipids have been recognized as receptors for *Helicobacter pylori*. The receptor structure has been considered to be NeuNAc α 3Gal- or NeuNAc α 3Gal β 4Glc (sialyl-lactose) (US documents 5,883,079, 5,753,630 and 5,514,660 and, Mysore et al. 1999) or NeuNAc α 3Gal β 4GlcNAc (Johansson and Miller-Podraza 1998, Miller-Podraza et al., 1997). More recently
20 fucosylated variants such as sialyl-Lewis x, NeuNAc α 3Gal β 4(Fuc α 3)GlcNAc (sLex), and especially a difucosylated structure sialyl-dimeric Lewis x, NeuNAc α 3Gal β 4(Fuc α 3)GlcNAc β 3Gal β 4(Fuc α 3)GlcNAc (sdiLex) (Mahdavi et al., 2002), have been considered as *Helicobacter* ligands. When the structures are expressed on glycolipids sdiLex is orders of magnitude better receptor for *H. pylori* than sLex (Mahdavi et al., 2002). The present invention is directed to other larger, non-fucosylated poly-N-acetyllactosamine structures. The fucosylated and sialylated polylactosamines are good receptors for *H. pylori*. However, relatively high affinity epitopes can be represented on non-fucosylated poly-N-acetyllactosamines. The benefit of these structures is that there is no need to add fucose to the epitopes by
25 chemical synthesis with 5-10 extra steps. The addition of fucose would also be industrially difficult as enzymatic or fermentative synthesis of fucosylated glycans
30 has not been developed to commercial level yet.

35 The present invention further shows that NeuNAc α 3Gal β 4GlcNAc-structures need to comprise structure - β 4Glc(NAc) for effective *H. pylori* binding. In general, similar glycolipid epitopes with other linkages to NeuNAc α 3Gal β 3GlcNAc and NeuNAc α 3Gal β 3GalNAc were not binding structures which observation is in contrast to what has been suggested for the terminal NeuNAc α 3Gal-epitope.

The heptasaccharide glycolipid

NeuNAc α 3Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4Glc β Cer have been shown to bind *H. pylori* (Roche. et al. 2001, Johansson and Miller-Podraza 1998, Miller-Podraza et al.1997). The present invention shows that the non-fucosylated structure

5 is actually more effective receptor for *H. pylori* than shorter

NeuNAc α 3Gal β 4Glc(NAc) or sLex epitopes giving possibility to design lower cost high affinity inhibitors or diagnostic reagents for *H. pylori*. The present invention allows recognition of the sialylated polylactosamines as high affinity receptors among the known sialic acid comprising receptors. Furthermore, the present

10 invention is directed to longer chain polylactosamines represented by glycolipid

NeuNAc α 3Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4Glc β Cer and to branched polylactosamine structures such as presented by glycolipid

NeuNAc α 3Gal β 4GlcNAc β 3(NeuNAc α 3Gal β 4GlcNAc β 6)Gal β 4GlcNAc β 3Gal β 4Glc β Cer.

15

Previously the inventors have also disclosed type 2 lactosamine binding epitope "neolacto-binding", -GlcNAc β 3Gal β 4GlcNAc β - for *H. pylori*. The binding epitope is active on longer chain linear poly-N-acetyllactoamines such as

20 NeuNAc α 3Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4Glc β Cer, however, this binding specificity does not require the presence of sialic acid as the desialylated structures are active (non-sialic acid binding strain) as well as N-glycolyl-neuraminic acid comprising structure NeuNGc α 3Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4Glc β Cer (FI 20010118), which is not recognized by the sialic acid binding specificity according to the present invention. When the common neolacto epitope was

25 characterized by not-sialic acid binding *H. pylori* strains, binding to

NeuNAc α 3Gal β 4GlcNAc β 3Gal β 4Glc β Cer was also not observable (FI20010118).

The sialic acid binding specificity is less common than the neolacto-binding specificity, but the sialic acid binding specificity is probably inflammation related and present in fresh pathologic isolates (Mahdavi et al., 2002). The branching of

30 polylactosamine chain can prevent binding to neolacto epitope but not to the high affinity sialic acid receptor according to the invention (FI20010118).

The prior art has also described a *H. pylori* binding protein called HPNAP with potential function in neutrophil activation. The present data shows that NAP-protein with sialic acid binding activity is not involved in inhibition of *H. pylori* binding to

35 sialylated structures. The potential function of the sialic acid binding of NAP-protein also including binding to NeuNAc α 3Gal β 4GlcNAc β 3Gal-type structures including longer polylactosamines may be related to interactions of *H. pylori* and neutrophils but its specificity and biological importance is not clear (Teneberg et al., 1997, Teneberg et al., 2000).

The prior art has also described non-sialylated type one epitopes: lactosylceramide Gal β 4Glc β Cer, gangliotetraosylceramide, Gal β 3GalNAc β 4Lac β Cer, (Lingwood et al., 1992), lacto-structures comprising terminal Gal β 3GlcNAc β - (Teneberg et al. 2002) and Lewis b Fuco α 2Gal β 3(Fuco α 4)GlcNAc β - (Borén et al, 1993). These are

- 5 based on different carbohydrate backbones: lactose, type 1 N-acetyllactosamines and ganglio-core and are clearly different in primary and three-dimensional structures from the sialylated longer type 2 N-acetyllactosamines according to the present invention. Moreover the references cited indicates that there are clearly specific strains for the different receptors and in the case of the sialic acid (sLex) binding
- 10 specificity (Mahdavi et al. 2002) and Lewis b-binding the very specific receptor proteins have been actually characterized (Ilver D., et al. 1998).

BRIEF DESCRIPTION OF THE DRAWINGS

- 15 **FIG. 1.** Negative ion FAB mass spectrum of the ganglioside from human erythrocytes. Above the spectrum is a simplified formula for interpretation, representing the species with sphingosine and non-hydroxy 24:0 fatty acid. The analysis was done as described under "Experimental procedures". Two spectra were collected, one using an acceleration voltage of 10 kV (A), and a second using an
- 20 acceleration voltage of 8 kV (B). For spectrum A a range of 100-2400 mass units was scanned, while for spectrum B, a range of 2000-3200 mass units were scanned and data was collected for 2.4 min.

- 25 **FIG. 2.** Electron ionisation mass spectrum of the permethylated ganglioside from human erythrocytes. Above the spectrum is a simplified formula for interpretation, representing the species with sphingosine and non-hydroxy 24:0 fatty acid. The analysis was done as described under "Experimental procedures". The spectrum was recorded at 380 °C. The peak at m/z 354 is due to a contaminant.

- 30 **FIG. 3.** Mass spectrum obtained by electrospray ionisation and collision induced dissociation of the permethylated ganglioside from human erythrocytes. Above the spectrum is a simplified formula for interpretation, representing the species with sphingosine and non-hydroxy 24:0 fatty acid. The analysis was done as described in the "Experimental procedures" section.

- 35 **FIG. 4.** Electron ionisation mass spectrum of the permethylated and reduced ganglioside from human erythrocytes. Above the spectrum is a simplified formula for interpretation, representing the species with sphingosine and non-hydroxy 24:0

fatty acid. The analysis was done as described under "Experimental procedures". The spectrum was recorded at 340 °C.

FIG. 5. Proton NMR spectrum at 600 MHz of the ganglioside from human erythrocytes (30 °C). The sample was dissolved in dimethyl sulphoxide/D₂O (98:2, by volume) after deuterium exchange. The broad peak(s) centred around 4.8 ppm (indicated by an *) represents a contaminant of unknown origin.

FIG. 6. Binding of *H. pylori* to NeuAc- and NeuGc-terminated gangliosides. Chemical detection by anisaldehyde (A), and autoradiograms obtained by binding of ³⁵S-labeled *H. pylori* strains CCUG 17874 (B) and J99 (C). The gangliosides were separated on aluminum-backed silica gel plates, using chloroform/metanol/0.25% KCl in water (50:40:10, by volume) as solvent system, and the binding assay was performed as described under "Experimental procedures". The *lanes* were:

15 NeuAc α 3Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer (NeuAc-neolactohexaocylceramide) of human hepatoma, 2 μ g (*lane 1*);
 NeuGc α 3Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer (NeuGc-neolactohexaocylceramide) of rabbit thymus, 2 μ g (*lane 2*); NeuAc α 3Gal β 4GlcNAc β 6 (NeuAc α 3Gal β 4GlcNAc β 3)Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer (NeuAc-G-10
 20 ganglioside) of human erythrocytes, 2 μ g (*lane 3*); NeuGc α 3Gal β 4GlcNAc β 6 (NeuGc α 3Gal β 4GlcNAc β 3)Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer (NeuGc-G-10
 ganglioside) of bovine erythrocytes, 2 μ g (*lane 4*); Gal α 3Gal β 4GlcNAc β 6 (NeuGc α 3Gal β 4GlcNAc β 3)Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer of bovine erythrocytes, 2 μ g (*lane 5*); Gal α 3(Fuc α 2)Gal β 4GlcNAc β 6
 25 (NeuAc α 3Gal β 4GlcNAc β 3)Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer (G9-B ganglioside) of human erythrocytes, 2 μ g (*lane 6*). Autoradiography was for 12 h.

FIG. 7. Comparison of binding of *H. pylori* strains CCUG 17874, J99. Chemical detection by anisaldehyde (A). Autoradiograms obtained by binding of ³⁵S-labeled *H. pylori* strain CCUG 17874 (B), and strain J99 (C).

The gangliosides were separated on aluminum-backed silica gel plates, using chloroform/metanol/0.25% KCl in water (50:40:10, by volume) as solvent system, and the binding assays were performed as described in the "Experimental procedures" section. The *lanes* were: gangliosides of human neutrophil granulocytes, 20 μ g (*lane 1*);
 35 NeuGc α 3Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer (NeuGc-neolactohexaocylceramide) of rabbit thymus, 2 μ g (*lane 2*);
 NeuAc α 3Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer (NeuAc-neolactohexaocylceramide) of human hepatoma, 1 μ g (*lane 3*);
 NeuAc α 3Gal β 4GlcNAc β 6

(NeuAc α 3Gal β 4GlcNAc β 3)Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer (NeuAc-G-10 ganglioside) of human erythrocytes, 1 μ g (*lane 4*); Gal α 3(Fuc α 2)Gal β 4GlcNAc β 6 (NeuAc α 3Gal β 4GlcNAc β 3)Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer (G9-B ganglioside) of human erythrocytes, 1 μ g (*lane 5*); Gal α 3(Fuc α 2)Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer (B6 type 2 hexaglycosylceramide) of human erythrocytes, 4 μ g (*lane 6*); Gal β 3GalNAc β 4Gal β 4Glc β 1Cer (gangliotetraosylceramide) of mouse feces, 4 μ g (*lane 7*). Autoradiography was for 12-24 h.

FIG. 8. Binding of *H. pylori* to serial dilutions of gangliosides. (A) Autoradiogram obtained by binding of *H. pylori* strain CCUG 17874 using the chromatogram binding assay. *Lanes 1-7* were serial dilutions (1-100 pmole) of NeuAc α 3Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer (NeuAc α 3-neolactotetraosylceramide), NeuAc α 3Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer (NeuAc α 3-neolactohexaacylceramide), and 15 NeuAc α 3Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer (NeuAc α 3- neolactooctaacylceramide), and *lane 8* was NeuAc α 3Gal β 3(Fuc α 4)GlcNAc β 3Gal β 4Glc β 1Cer (sialyl-Le a hexaglycosylceramide), 1 mmole. The binding assay was done as described under "Experimental procedures". The results from one representative experiment out of three is shown. (B) 20 Quantification of binding by densitometry. The autoradiogram in (A) was analyzed using the NIH Image program.

FIG. 9. Binding of *H. pylori* to serial dilutions of gangliosides. (A) Autoradiogram obtained by binding of *H. pylori* strain 17874 using the chromatogram binding assay. 25 The *lanes* were: serial dilutions of NeuAc α 3Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer (NeuAc-neolactohexaacylceramide), (10-100 pmole) (*lanes 1-5*); serial dilutions of NeuAc α 3Gal β 4GlcNAc β 3Gal β 4(Fuc α 3)GlcNAc β 3Gal β 4Glc β 1Cer (VIM-2 ganglioside), (10-100 pmole) (*lanes 6-10*); serial dilutions of 30 NeuAc α 3Gal β 4(Fuc α 3)GlcNAc β 3Gal β 4(Fuc α 3)GlcNAc β 3Gal β 4Glc β 1Cer (NeuAc-dimeric-Le x ganglioside), (10-100 pmole) (*lanes 11-14*); NeuAc α 3Gal β 4(Fuc α 3)GlcNAc β 3Gal β 4Glc β 1Cer (sialyl-Le x hexaglycosylceramide), 1 mmole (*lane 15*). The binding assay was done as described under "Experimental procedures". The results from one representative experiment out of three is shown. (B) 35 Quantification of binding by densitometry. The autoradiogram in (A) was analyzed using the NIH Image program.

FIG. 10. Binding of *H. pylori* to serial dilutions of gangliosides. (A)

Autoradiogram obtained by binding of *H. pylori* strain CCUG 17874 to serial dilutions (1-100 pmole) of NeuAc α 3Gal β 4(Fuca3)GlcNAc β 3Gal β 4Glc β 1Cer (NeuAc-Le x), NeuAc α 3Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer (NeuAc α 3-neolactohexaocylceramide),

5 NeuAc α 3Gal β 4GlcNAc β 6(NeuAc α 3Gal β 4GlcNAc β 3)Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer (NeuAc-G-10 ganglioside) and
Gal α 3(Fuca2)Gal β 4GlcNAc β 6(NeuAc α 3Gal β 4GlcNAc β 3)Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer (G9-B ganglioside) using the chromatogram binding assay. The binding assay was done as described under "Experimental procedures". The results from one

10 representative experiment out of three is shown. (B) Quantification of binding by densitometry. The autoradiogram in (A) was analyzed using the NIH Image program. For comparison, the result densitometry of the binding of 125 I-labeled cholera toxin B-subunits (CTB) to dilutions of the GM1 ganglioside on a thin-layer chromatogram is included in (A).

15

Fig. 11 Detection level of granulocyte gangliosides on TLC plates using radiolabeled *H. pylori*. The plates were developed in C/M/0.25%KCl in water, 50:40:10, and visualized by spraying with anisaldehyde (Anis) or by overlay with 35 S-labeled *H. pylori*, CCGU 17874. Lanes 1-10, two-fold dilutions of granulocyte gangliosides (total 12 μ g in Lane 1). Lane 11, bovine brain gangliosides, 2 μ g (mixture of GM1, GD1a, GD1b and GT1b); S-3PG and S-6PG stand for NeuAc α 3Gal β 4GlcNAc β 3Gal β 4GlcCer and NeuAc α 6Gal β 4GlcNAc β 3Gal β 4GlcCer, respectively. Annotations 7s and 8s indicate chromatographic regions containing gangliosides with 7 and 8 monosaccharides per molecule.

Fig. 12. Negative ion FAB spectra of S-3PG derivatised at the -CH₂CH₂CH₂OH and -COOH groups of the sialic acid. **Panel A**, S-3PG, unmodified; **Panel B**, oxidized/reduced; **Panel C**, After oxidation and derivatization with methylamine; **Panel D**, After oxidation and derivatization with ethanolamine. **Panel E**, reduced at -COOH; **Panel F**, free amide; **Panel G**, Methylamide; **Panel H**, Ethylamide; **Panel I**, Propylamide; **Panel J**, benzylamide; **Panel K**, Octadecylamide

35 Fig. 13. Example of binding of *H. pylori* (CCGU 17874) to modified S-3PG. Glycolipids were separated on TLC plates and visualized with anisaldehyde (Anis, left plate) or with 35 S-labeled bacterium (*H. pylori*, right plate). For chromatographic conditions see Fig. 1. **Lane 1**, S-3PG. This lane also contains trace amounts of longer members of the binding series; **Lane 2**, ethylamide of S-3PG;

Lane 3, propylamide of S-3-PG; **Lane 4**, benzylamide of S-3-PG; **Lane 5**, oxidized/reduced S-3-PG, see Table 3; **Lane 6**, positive control for *H. pylori*; **Lane 7**, bovine brain gangliosides (from top: GM1, GD1a, GD1b, GT1b); **Lane 8**, mixture of sulfatides; **Lane 9**, mixture of five sugar-containing gangliosides from rabbit thymus; **Lane 10**, mixture of complex gangliosides prepared from human granulocytes.

Fig. 14. Binding of *H. pylori* (CCGU 17874 strain) to neoglycolipids on TLC plates.

For chromatographic conditions see Fig. 1. Left plate was stained for carbohydrates by anisaldehyde and the right plate was overlaid with ^{35}S -labeled *H. pylori*.

Lane 1, S-3PG. Like in Fig. 3, the SPG preparation contains trace amounts of longer members of the binding series; **Lane 2**, neoglycolipids formed from NeuAc α 3Gal β 4GlcNAc β 3Gal β 4Glc; **Lane 3**, neoglycolipids formed from NeuAc α 3Gal β 3GlcNAc β 3Gal β 4Glc; **Lane 4**, neoglycolipids formed from NeuAc α 6Gal β 4GlcNAc β 3Gal β 4Glc; **Lane 5**, neoglycolipids formed from Gal β 3(NeuAc α 6)GlcNAc β 3Gal β 4Glc;

Lane 6, bovine brain gangliosides (from top: GM1, GD1a, GD1b, GT1b). Arrows in lane 2 indicate hexadecylaniline-derivative (lower band) and neoglycolipid with branched lipid chain (upper double band). The marked fractions in lane 2 and the corresponding fractions in lanes 3-5 were scraped off and tested by mass spectrometry, see Fig. 6.

Fig. 15. Negative ion FAB spectra of neoglycolipids derived from NeuAc α 3Gal β 4GlcNAc β 4Gal β 4Glc. **Panel A**, hexadecylaniline derivative; **Panel B**, neoglycolipid with branched lipid part.

Fig. 16. Example of EI MS (electron ionization mass spectrometry) of permethylated polyglycosylceramides showing fragment ions corresponding to NeuAc and the reduced NeuAc.

Fig. 17. Binding of *H. pylori* (032 strain) to derivatized PGCs on TLC plates. Left plate was stained for carbohydrates by anisaldehyde and the right plate was overlaid with ^{35}S -radiolabeled *H. pylori*. **Lanes 1 and 2**, underivatized PGCs of human erythrocytes; **Lane 3**, reduced PGCs (COOH \rightarrow CH₂OH); **Lane 4**, bovine brain gangliosides (mixture of GM1, GD1a, GD1b, GT1b); **Lane 5**, S-3PG. (Note that bacteria 032 from broth do not bind to S-3PG which represent the linear structure).

DETAILED DESCRIPTION OF THE INVENTION

The present invention shows that several linear and branched NeuNAc α 3-poly-N-acetyllactosamine structures can serve as high affinity ligands for *Helicobacter pylori*. The binding is specific for NeuNAc α 3 linked to the type lactosamine Gal β 4GlcNAc. The present invention is directed to larger polylactosamines having higher binding activity than the terminal trisaccharide epitope. When considering the linear polylactosamine structures NeuNAc α 3Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4Glc β Cer and 10 NeuNAc α 3Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4Glc β Cer it is clear that the presentation of the terminal NeuNAc α 3Gal β 4GlcNAc is effective on β 3Gal β 4GlcNAc and larger polylactosamine β 3Gal β 4GlcNAc β 3Gal β 4GlcNAc. The results also indicate that the terminal structure β 3-linked on Gal or lactose are useful minimal epitopes.

15

General formula of novel high affinity inhibitors of *H. pylori*

The present invention is specifically directed to high affinity *Helicobacter pylori* binding oligosaccharide sequences according to the Formula 1:

20 R₁Gal β 4GlcNAc β 3 { (R₂Gal β 4GlcNAc β 6) }_{s1} Gal[β 4GlcNAc β 3Gal] _{s2} { β 4Glc[NAc] _{s3} } _{s4}

wherein R₁ and R₂ are independently nothing, or terminal mono-or oligosaccharides substituents with the proviso that at least one of the substituents is NeuNAc α 3 or NeuNAc α 3Gal β 4GlcNAc β 3. Integers s₁, s₂, s₃ and s₄ are independently 0 or 1, 25 indicating the presence or absence of the structures in [] or in { }. The possible branch in the structure is marked as (). The present invention is also directed to structural analogs, especially conformational analogs, or derivatives of said oligosaccharide sequence having binding activity to *Helicobacter pylori*

30 Structure of potential non-sialylated branch structure

Preferably R₁ or R₂, when not being NeuNAc α 3, indicates terminal substituents linked to position 2 and/or 3 of the terminal Gal according to Formula 2

Hex[NAc]_{t1} α / β 3[(DeoxyHex α 2)]_{t2}

35

wherein Hex is preferably Gal or Glc. Integers t₁ and t₂ are independently 0 or 1. α/β means that the linkage is either α or β .

Preferably non-sialylated R1 or R2 is a structure selected from the group consisting of Gal α 3, GalNAc α 3, Fuca2, Gal α 3(Fuca2), GalNAc α 3(Fuca2), NeuNAc α 3Gal β 4GlcNAc β 3, Gal β 4GlcNAc β 3, GlcNAc β 3Gal β 4GlcNAc β 3, GlcNAc α 3, GlcNAc β 3, GalNAc β 3, Gal β 3, Glc β 3, and Glc α 3. More preferably the 5 structure is selected from the group of blood group antigen like structures: Gal α 3, GalNAc α 3, Fuca2, Gal α 3(Fuca2), and GalNAc α 3(Fuca2).

Preferred branched structures

In a preferred embodiment s1 is 1 and both R1 and R2 are selected from the group 10 consisting of NeuNAc α 3 or NeuNAc α 3Gal β 4GlcNAc β 3. The preferred poly-N-acetyl lactosamine structures include oligosaccharide sequences
 NeuNAc α 3LacNAc β 3(NeuNAc α 3LacNAc β 6)LacNAc β 3LacNAc
 NeuNAc α 3LacNAc β 3(NeuNAc α 3LacNAc β 3LacNAc β 6)LacNAc
 NeuNAc α 3LacNAc β 3LacNAc β 3(NeuNAc α 3LacNAc β 6)LacNAc
 15 NeuNAc α 3LacNAc β 3(NeuNAc α 3LacNAc β 6)LacNAc β 3Lac
 NeuNAc α 3LacNAc β 3(NeuNAc α 3LacNAc β 6)LacNAc β 3Gal
 NeuNAc α 3LacNAc β 3(NeuNAc α 3LacNAc β 6)Lac
 NeuNAc α 3LacNAc β 3(NeuNAc α 3LacNAc β 6)LacNAc
 NeuNAc α 3LacNAc β 3(NeuNAc α 3LacNAc β 6)Gal

20 The oligosaccharide sequences according to the present invention may be further presented as branched poly-N-acetyllactosamines, for example

NeuNAc α 3LacNAc β 3(NeuNAc α 3LacNAc β 6)LacNAc β 3(NeuNAc α 3LacNAc β 6)LacNAc

25 wherein LacNAc indicates N-acetyllactosamine, Gal β 4GlcNAc, and Lac is lactose, Gal β 4Glc, the two sequences below indicate the same structures:

NeuNAc α 3Gal β 4GlcNAc β 3(NeuNAc α 3Gal β 4GlcNAc β 6)Gal β 4Glc

NeuNAc α 3LacNAc β 3(NeuNAc α 3LacNAc β 6)Lac

30 Preferred long chain lactosamine epitopes

When s1 is 0 the Formula 1 describes linear sialylpolylactosamines such as

NeuNAc α 3LacNAc β 3LacNAc β 3LacNAc

NeuNAc α 3LacNAc β 3LacNAc β 3Lac

35 NeuNAc α 3LacNAc β 3LacNAc β 3Gal

NeuNAc α 3LacNAc β 3LacNAc

And shorter minimal epitopes:

NeuNAc α 3LacNAc β 3Lac

NeuNAc α 3LacNAc β 3Gal

Preferred analog structures

The present invention shows that the glycerol tail of the NeuNAc residue does not tolerate modifications produced by the oxidation and reduction. Acetyl group of NeuNAc does not tolerate much changes, thus glycolyl-, propyl-, or deacetylated

5 amine analogs are weakly active or not active at all. Also the variants in which the carboxylic acid group of the sialic acid is reduced to alcohol are not active. The similar specificities were shown with small pentasaccharide epitopes and branched large polyglycosylceramides. Positioning of the sialic acid residue is also important, disialic acid or NeuNAc α 6-structures were not active. Furthermore the terminal N-
10 acetyllactosamine to which the NeuNAc-residue is α 3-linked should be type two N-acetyllactosamine Gal β 4GlcNAc, while type 1 N-acetyllactosamine, Gal β 3GlcNAc, or asialo-ganglioside terminals, Gal β 3GalNAc are not accepted. The data allows effective design of tolerable analogs and derivatives avoiding the non-active structural features.

15

The present invention is especially directed to analogs of the structures according to the Formula 1 wherein at least one of N-acetyllactosamine residues have been replaced by type 2 N-acetyllactosamine analogous structure or structures, preferably by lactose residues according to Formula 3

20

R₁Gal β 4Glc[NAc]_{u1} β 3{(R₂Gal β 4Glc[NAc]_{u2} β 6)}_{s1}Gal{ β 4Glc[NAc]_{u3} β 3Gal}_{s2}{ β 4Glc[NAc]_{s3}}_{s4}

wherein R1 and R2 are independently nothing or terminal mono- or oligosaccharides substituents with the proviso that at least one of the substituents is NeuNAc α 3 or

25 NeuNAc α 3Gal β 4Glc[NAc]_{u4} β 3. Integers s1, s2, s3 and s4 are independently 0 or 1, indicating the presence or absence of the structures in [] or in {}. Integers u1, u2, u3, and u4 are independently 0 or 1, indicating the presence of absence of the N-acetyl groups in the non-reducing end terminal or midchain lactosamine residues with the proviso that at least one of the integers present is 0. Type 2 N-acetyllactosamine

30 analogous structures described herein include Gal β 4Glc2-X structures in which carbon 2 of Glc-ring is linked to group X, which is preferably -NH₂ (Gal β GlcN-analog) or N-alkyl (Gal β 4GlcN-alkyl) or N-alkanoyl. A preferred alkanoyl is propanoyl. Carbon 2 may also be derivatized by an O-ester such as O-acetyl or O-ether such as O-methyl. Especially disialylated and linear monosialylated structures

35 are preferred:

NeuNAc α 3Lac[NAc]_{u1} β 3(NeuNAc α 3Lac[NAc]_{u2} β 6)Lac[NAc]_{u3} β 3Gal{ β 4Glc[NAc]_{s3}}_{s4}

NeuNAc α 3Lac[NAc]_{u1} β 3(NeuNAc α 3Lac[NAc]_{u2} β 3Lac[NAc]_{u3} β 6)Gal{ β 4Glc[NAc]_{s3}}_{s4}

NeuNAc α 3Lac[NAc]_{u1} β 3Lac[NAc]_{u2} β 3(NeuNAc α 3Lac[NAc]_{u3} β 6)Gal{ β 4Glc[NAc]_{s3}}_{s4}



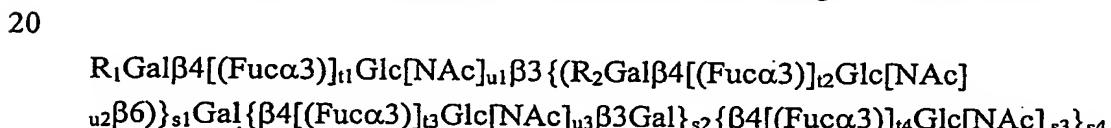
The invention is also directed to analogs according to structure

5 NeuNAc α 3Lac[NAc] $_{u1}$ β 3Lac[NAc] $_{u2}$
with the proviso that when u2 is 0 then u1 is also 0.

Fucosylated analogs

As discussed above the fucosylated structures such as sLex and sdiLex have been
10 studied separately. The present invention shows that the same binding specificity is involved with both fucosylated and non-fucosylated sialyl α 3-N-acetyllactosamines. It is realized that the lactosamine analogs of the poly-N-acetyllactosamine described above, especially the lactose comprising analogs would also be active when α 3-fucosylated to Glc and (if also present) to GlcNAc residues. Such analogs would
15 combine cheaper backbone structures with higher affinity induced by the fucose residues. The present invention is directed to the sialylated and fucosylated structures as such.

The most preferred fucosylated structures are according to the formula 4



wherein R1 and R2 are independently nothing or terminal mono- or oligosaccharides
25 substituents with the proviso that at least one of the substituents is NeuNAc α 3 or NeuNAc α 3Gal β 4[(Fuc α 3)] $_{t5}$ Glc[NAc] $_{u4}\beta3$. Integers s1, s2, s3, and s4 are independently 0 or 1, indicating the presence or absence of the structures in [] or in {}. Integers u1, u2, u3, and u4 are independently 0 or 1, indicating the presence of absence of the N-acetyl groups in the non-reducing end terminal or midchain
30 lactosamine residues with the proviso that at least one of the integers present is 0. Integers t1, t2, t3, t4 and t5 are independently 0 or 1, indicating the presence or absence of the Fuc α 3-branch-structures in []. Especially disialylated and linear monosialylated structures are preferred.

35 The present invention is also directed to non-fucosylated and fucosylated analog structures comprising N-acetyllactosamine analogue at non-reducing position. Especially, analogs comprising lactose are preferred, wherein at least one of u1, u2 or u3 is 0, more preferably two of the variables are 0 and most preferably all three are 0. In a preferred embodiment the analog is branched and more preferably

disialylated. Separately, pentasaccharide, hexasaccharide and heptasaccharide structures are preferred as analog substances according to the invention.

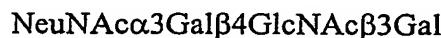
Minimal epitopes giving better presentation of NeuNAc α 3Gal β 4Glc(NAc)

- 5 The structure close to ceramide is sterically more restricted in the TLC binding assay. As a free monovalent inhibitor or as polyvalent conjugate the oligosaccharide sequences NeuNAc α 3Gal β 4GlcNAc β 3Gal β and NeuNAc α 3Gal β 4GlcNAc β 3Gal β 4Glc and especially NeuNAc α 3Gal β 4GlcNAc β 3Gal β 4GlcNAc are more active than the sialylactose or
- 10 10 NeuNAc α 3Gal β 4GlcNAc. The use of neoglycolipid structure demonstrated that reductively aminated structure NeuNAc α 3Gal β 4GlcNAc β 3Gal β 4Glc-hexadecylaniline is active even though the reducing end glucose is not in ring form after the reductive conjugation. The activity was similar to the glycolipid structure NeuNAc α 3Gal β 4GlcNAc β 3Gal β 4Glc β Cer when the trisaccharide glycolipid
- 15 15 NeuNAc α 3Gal β 4Glc β Cer (GM3) was inactive.

The present invention is specifically directed to functional foods, especially infant foods including infant formulas and food additives comprising the added or enriched sialylated polylactosamines according to the present invention which are present in 20 human and animal milks. Especially preferred structures for the food uses include one or several oligosaccharides selected from the group NeuAc α 3Gal β 4GlcNAc β 3Gal β 4Glc, NeuNAc α 3Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4Glc, NeuNAc α 3Gal β 4GlcNAc β 3(NeuNAc α 3Gal β 4GlcNAc β 6)Gal β 4Glc and more 25 preferably the structures for functional foods include NeuAc α 3Gal β 4GlcNAc β 3Gal β 4Glc and/or NeuNAc α 3Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4Glc and most preferably NeuAc α 3Gal β 4GlcNAc β 3Gal β 4Glc. The present invention is further directed to oligosaccharide composition comprising the three oligosaccharides or any 30 combinations of two oligosaccharides selected from the above group in essentially pure form (essentially pure oligosaccharide fraction comprise at least 80 carbohydrate mass % of the desired oligosaccharide or oligosaccharides, more preferably the essentially pure fraction comprises at least 90 % and most preferably at least 95 % of the desired oligosaccharides) and use of the compositions in 35 preparation of functional foods or pharmaceutical or therapeutic compositions. The present invention is further directed to the use of the oligosaccharide sequences in chewing gums and various consumer products.

In another embodiment of the invention it is also preferred to have a sialylated oligosaccharide from the above group or 2 or 3 of the saccharides as an essentially pure mixture with one or several oligosaccharides selected from the group consisting of human milk or animal milk saccharides such as sialyl lactose(s), lactose, lacto-N-neotetraose, para-lacto-N-neotetraose, lacto-N-neohexaose and α 3-fucosylated derivates thereof. A preferred sialic oligosaccharide mixture contains NeuNAc α 3Lac, NeuNAc α 3LacNAc β 3Lac and NeuNAc α 3(LacNAc β 3) $_2$ Lac or more preferably such mixture contains only the two first mentioned.

10 The present invention is especially directed to minimal higher affinity receptor oligosaccharide sequence according to the formula 5



15 with the proviso that the structure is not presented as a pentasaccharide glycolipid NeuNAc α 3Gal β 4GlcNAc β 3Gal β 4Glc β Cer. More preferably the oligosaccharide sequence is not linked to ceramide or a hydrophobic aglycon or spacer comprising more than 22 carbon atoms. More preferably the tetrasaccharide sequence is coupled to an aglycon or spacer comprising less than 8 carbon atoms in a hydrophobic
20 structure.

The present invention is also directed to the conformational analogs and derivative of the above structures. Preferably the structural analog or derivative structures have similar or better affinity towards *H. pylori*. In a preferred class of analogs the analog
25 comprise lactose or another disaccharide epitope having similar conformation with type 2 N-acetyllactosamine. Preferred analog structures include oligosaccharide sequences NeuNAc α 3Gal β 4Glc β 3Gal. The present invention is specifically directed to substances comprising terminal oligosaccharide sequence NeuNAc α 3Gal β 4Glc β 3Gal. The lactose based structures are much cheaper to
30 produce chemically than N-acetyllactosamine structures.

The present invention is especially directed to minimal higher affinity receptor oligosaccharide sequence according to the formula 6



wherein m is 0 or 1 with the proviso that the structure is not presented as a pentasaccharide glycolipid NeuNAc α 3Gal β 4GlcNAc β 3Gal β 4Glc β Cer. More preferably the oligosaccharide sequence is not linked to ceramide or a hydrophobic

aglycon or spacer comprising more than 22 carbon atoms. More preferably the tetrasaccharide sequence is coupled to an aglycon or spacer comprising less than 8 carbon atoms in a hydrophobic structure. The minimal higher affinity sequences are especially useful in polyvalent conjugates and as free saccharides.

5

The present invention is also directed to the conformational analogs and derivative of the structures. Preferably the structural analog or derivative structures have similar or better affinity towards *H. pylori*. In a preferred class of analogs the analog comprise lactose or another disaccharide epitope having similar conformation with type 2 N-acetyllactosamine. Preferred analog structures include oligosaccharide sequences NeuNAc α 3Gal β 4Glc β 3Gal β 4Glc(NAc)_m wherein m is 0 or 1. The present invention is specifically directed to substances comprising terminal oligosaccharide sequence NeuNAc α 3Gal β 4Glc β 3Gal β 4Glc or NeuNAc α 3Gal β 4Glc β 3Gal β 4GlcNAc. The lactose based structures are much cheaper to produce chemically than N-acetyllactosamine structures.

Novel neutral sialic acid derivatives having binding activities towards *H. pylori* than other sialylated polylactosamine structures

20

The present invention further describes novel sialic acid derivatives with binding affinity towards *Helicobacter pylori*. The derivatives have the structure

SA(X-R)

25

wherein X is a linking atom or group bound to C1 of sialic acid, R is H or an organic radical comprising more than 3 carbon atoms. Preferably X is -NH forming amide structure with the carboxylic acid group of the sialic acid residue. Preferably R is H or a C₄- C₃₀ organic radical comprising a ring structure and/or an aliphatic chain. More preferably R is a C₆-C₂₄ organic radical and most preferably R is a C₆-24 aliphatic alkyl chain. Preferably the sialic acid is NeuNAc.

The invention is further directed to analogs and derivatives of the modified sialic acid structure.

35

More preferably the sialic acid structure is linked to lactose or N-acetyllactosamine structure. In a preferred embodiment the sialic acid derivative is α 3-linked to type two N-acetyllactosamine sequence:



wherein x is linkage position of the sialic acid derivative and integers p1, p2 and p3 are independently 0 or 1 indicating the presence or absence of

5 the whole structure in { }, [] or ().

It is further realized that the novel sialic acid derivatives can be presented on poly-N-acetyllactosamines as described for NeuNAc according to the invention and on other types of glycoconjugates with binding activity towards *H. pylori*.

10

It is realized that the neutral sialic acid epitopes can bind different receptors than acidic NeuNAc on the surface of *H. pylori*. The invention specifically aims for use of the novel receptor for identification of corresponding adhesin from the surface of *H. pylori*. The present invention is further directed to design of potential new ligands 15 inhibiting *H. pylori* binding to human and animal cells and tissues or for agglutination of the bacterium. The design and synthesis of the novel *H. pylori* binding substances is further directed to use of modeling with other carbohydrate structures binding the same receptor of the *H. pylori*.

20

In this invention the terms "analog" and "derivative" are defined as follows. According to the present invention it is possible to design structural analogs or derivatives of the *Helicobacter pylori* binding oligosaccharide sequences. Thus, the invention is also directed to the structural analogs of the substances according to the invention. The structural analogs according to the invention comprises the structural 25 elements important for the binding of *Helicobacter pylori* to the oligosaccharide sequences. For design of effective structural analogs it is necessary to know the structural element important for the binding between *Helicobacter pylori* and the saccharides. The important structural elements are preferably not modified or these are modified by a very close mimetic of the important structural element.

30

The structural derivatives according to the invention are oligosaccharide sequences according to the invention modified chemically so that the binding to the *Helicobacter pylori* is retained or increased. According to the invention it is preferred to derivatize one or several of the hydroxyl or acetamido groups of the 35 oligosaccharide sequences. The invention describes several positions of the molecules which could be changed when preparing the analogs or the derivatives. The hydroxyl or acetamido groups which preferably tolerate at least certain

modifications are self-evident for a skilled artisan from the formulas described herein.

Bulky or acidic substituents and other structures, such as monosaccharide residues,
5 are not tolerated, but methods to produce oligosaccharide analogs e.g. for the binding of a lectin are well known. For example, numerous analogs of sialyl-Lewis x oligosaccharide has been produced, representing the active functional groups different scaffold, see page 12090 Sears and Wong 1996. Similarly analogs of heparin oligosaccharides has been produced by Sanofi corporation and sialic acid
10 mimicking inhibitors such as Zanamivir and Tamiflu (Relenza) for the sialidase enzyme by numerous groups. Preferably the oligosaccharide analog is build on a molecule comprising at least one six- or five-membered ring structure, more preferably the analog contains at least two ring structures comprising 6 or 5 atoms. In mimicking structures monosaccharide rings may be replaced rings such as
15 cyclohexane or cyclopentane, aromatic rings including benzene ring, heterocyclic ring structures may comprise beside oxygen for example nitrogen and sulphur atoms. To lock the active ring conformations the ring structures may be interconnected by tolerated linker groups. Typical mimetic structure may also comprise peptide analog-structures for the oligosaccharide sequence or part of it.
20

The effects of the active groups to binding activity are cumulative and lack of one group could be compensated by adding an active residue on the other side of the molecule. Molecular modelling, preferably by a computer can be used to produce analog structures for the *Helicobacter pylori* binding oligosaccharide sequences
25 according to the invention. The results from the molecular modelling of several oligosaccharide sequences are given in examples and the same or similar methods, besides NMR and X-ray crystallography methods, can be used to obtain structures for other oligosaccharide sequences according to the invention. To find analogs the oligosaccharide structures can be "docked" to the carbohydrate binding molecule(s)
30 of *H. pylori*, most probably to lectins of the bacterium and possible additional binding interactions can be searched.

It is also noted that the monovalent, oligovalent or polyvalent oligosaccharides can be activated to have higher activity towards the lectins by making derivative of the
35 oligosaccharide by combinatorial chemistry. When the library is created by substituting one or few residues in the oligosaccharide sequence, it can be considered as derivative library, alternatively when the library is created from the analogs of the oligosaccharide sequences described by the invention. A combinatorial chemistry library can be built on the oligosaccharide or its precursor or on glycoconjugates

according to the invention. For example, oligosaccharides with variable reducing end can be produced by so called carbohydrid technology.

In a preferred embodiment a combinatorial chemistry library is conjugated to the *Helicobacter pylori* binding substances described by the invention. In a more preferred embodiment the library comprises at least 6 different molecules. Such library is preferred for use of assaying microbial binding to the oligosaccharide sequences according to the invention. A high affinity binder could be identified from the combinatorial library for example by using an inhibition assay, in which the library compounds are used to inhibit the bacterial binding to the glycolipids or glycoconjugates described by the invention. Structural analogs and derivatives preferred according to the invention can inhibit the binding of the *Helicobacter pylori* binding oligosaccharide sequences according to the invention to *Helicobacter pylori*.

In the following the *Helicobacter pylori* binding sequence is described as an oligosaccharide sequence. The oligosaccharide sequence defined here can be a part of a natural or synthetic glycoconjugate or a free oligosaccharide or a part of a free oligosaccharide. Such oligosaccharide sequences can be bonded to various monosaccharides or oligosaccharides or polysaccharides on polysaccharide chains, for example, if the saccharide sequence is expressed as part of a bacterial polysaccharide. Moreover, numerous natural modifications of monosaccharides are known as exemplified by O-acetyl or sulphated derivative of oligosaccharide sequences. The *Helicobacter pylori* binding substance defined here can comprise the oligosaccharide sequence described as a part of a natural or synthetic glycoconjugate or a corresponding free oligosaccharide or a part of a free oligosaccharide. The *Helicobacter pylori* binding substance can also comprise a mix of the *Helicobacter pylori* binding oligosaccharide sequences.

The *Helicobacter pylori* binding substances may be part of a saccharide chain or a glycoconjugate or a mixture of glycocompounds containing other known *Helicobacter*

30 binding epitopes, with different saccharide sequences and conformations, such as Lewis b, Fuc α 2Gal β 3(Fuc α 4)GlcNAc, or Neu5Aca3Gal β 4Glc/GlcNAc. Using several binding substances together may be beneficial for therapy.

The *Helicobacter pylori* binding oligosaccharide sequences can be synthesized enzymatically by glycosyltransferases, or by transglycosylation catalyzed by glycosidase or transglycosidase enzymes (Ernst *et al.*, 2000). Specificities of these enzymes and the use of co-factors can be engineered. Specific modified enzymes can be used to obtain more effective synthesis, for example, glycosynthase is modified to do transglycosylation only. Organic synthesis of the saccharides and the conjugates described herein or compounds

similar to these are known (Ernst *et al.*, 2000). Saccharide materials can be isolated from natural sources and modified chemically or enzymatically into the *Helicobacter pylori* binding compounds. Natural oligosaccharides can be isolated from milks produced by various ruminants. Transgenic organisms, such as cows or microbes, expressing glycosylating enzymes can be used for the production of saccharides.

The bacterium binding substances are preferably represented in clustered form such as by glycolipids on cell membranes, micelles, liposomes, or on solid phases such as TCL-plates used in the assays. The clustered representation with correct spacing creates high affinity binding.

According to the invention it is also possible to use the *Helicobacter pylori* binding epitopes or naturally occurring, or a synthetically produced analogue or derivative thereof having a similar or better binding activity with regard to *Helicobacter pylori*.
15 It is also possible to use a substance containing the bacterium binding substance such as a receptor active ganglioside described in the invention or an analogue or derivative thereof having a similar or better binding activity with regard to *Helicobacter pylori*. The bacterium binding substance may be a glycosidically linked terminal epitope of an oligosaccharide chain. Alternatively the bacterium binding
20 epitope may be a branch of an oligosaccharide chain, preferably a polylactosamine chain.

The *Helicobacter pylori* binding substance may be conjugated to an antibiotic substance, preferably a penicillin type antibiotic. The *Helicobacter pylori* binding substance targets the antibiotic to *Helicobacter pylori*. Such conjugate is beneficial in treatment because a lower amount of antibiotic is needed for treatment or therapy against *Helicobacter pylori*, which leads to lower side effect of the antibiotic. The antibiotic part of the conjugate is aimed at killing or weaken the bacteria, but the conjugate may also have an antiadhesive effect as described below.

30 The bacterium binding substances, preferably in oligovalent or clustered form, can be used to treat a disease or condition caused by the presence of the *Helicobacter pylori*. This is done by using the *Helicobacter pylori* binding substances for anti-adhesion, i.e. to inhibit the binding of *Helicobacter pylori* to the receptor epitopes of
35 the target cells or tissues. When the *Helicobacter pylori* binding substance or pharmaceutical composition is administered it will compete with receptor glycoconjugates on the target cells for the binding of the bacteria. Some or all of the bacteria will then be bound to the *Helicobacter pylori* binding substance instead of the receptor on the target cells or tissues. The bacteria bound to the *Helicobacter*

pylori binding substances are then removed from the patient (for example by the fluid flow in the gastrointestinal tract), resulting in reduced effects of the bacteria on the health of the patient. Preferably the substance used is a soluble composition comprising the *Helicobacter pylori* binding substances. The substance can be

5 attached to a carrier substance which is preferably not a protein. When using a carrier molecule several molecules of the *Helicobacter pylori* binding substance can be attached to one carrier and inhibitory efficiency is improved.

The target cells are primarily epithelial cells of the target tissue, especially the
10 gastrointestinal tract, other potential target tissues are for example liver and pancreas. Glycosylation of the target tissue may change because of infection by a pathogen (Karlsson *et al.*, 2000). Target cells may also be malignant, transformed or cancer/tumour cells in the target tissue. Transformed cells and tissues express altered types of glycosylation and may provide receptors to bacteria. Binding of lectins or

15 saccharides (carbohydrate-carbohydrate interaction) to saccharides on glycoprotein or glycolipid receptors can activate cells, in case of cancer/malignant cells this may be lead to growth or metastasis of the cancer. Several of the oligosaccharide epitopes and sialylated polylactosamines from malignant cells (Stroud *et al.*, 1996), have been reported to be cancer-associated or cancer antigens. *Helicobacter pylori* is

20 associated with gastric lymphoma. The substances described herein can be used to prevent binding of *Helicobacter pylori* to premalignant or malignant cells and activation of cancer development or metastasis. Inhibition of the binding may cure gastric cancer, especially lymphoma.

25 Target cells also includes blood cells, especially leukocytes. It is known that *Helicobacter pylori* strains associated with peptic ulcer, as the strain mainly used here, stimulates an inflammatory response from granulocytes, even when the bacteria are nonopsonized (Rautelin *et al.*, 1994a,b). The initial event in the phagocytosis of the bacterium most likely involves specific lectin-like interactions
30 resulting in the agglutination of the granulocytes (Ofek and Sharon, 1988).

Subsequent to the phagocytotic event oxidative burst reactions occur which may be of consequence for the pathogenesis of *Helicobacter pylori*-associated diseases (Babior, 1978). Several sialylated and non-acid glycosphingolipids having repeating N-acetyllactosamine units have been isolated and characterized from granulocytes
35 (Fukuda *et al.*, 1985; Stroud *et al.*, 1996) and may thus act as potential receptors for *Helicobacter pylori* on the white blood cell surface. Furthermore, also the X₂ glycosphingolipid has been isolated from the same source (Teneberg, S., unpublished). The present invention confirms the presence of receptor saccharides on human erythrocytes and granulocytes which can be recognized by an N-

acetyllactosamine specific lectin and by a monoclonal antibody (X_2 , GalNAc β 3Gal β 4GlcNAc-). The *Helicobacter pylori* binding substances can be useful to inhibit the binding of leukocytes to *Helicobacter pylori* and in prevention of the oxidative burst and/or inflammation following the activation of leukocytes.

5

It is known that *Helicobacter pylori* can bind several kinds of oligosaccharide sequences. Some of the binding by specific strains may represent more symbiotic interactions which do not lead to cancer or severe conditions. The present data about binding to cancer-type saccharide epitopes indicates that the *Helicobacter pylori* binding substance can prevent more pathologic interactions, in doing this it may leave some of the less pathogenic *Helicobacter pylori* bacteria/strains binding to other receptor structures. Therefore total removal of the bacteria may not be necessary for the prevention of the diseases related to *Helicobacter pylori*. The less pathogenic bacteria may even have a probiotic effect in the prevention of more pathogenic strains of *Helicobacter pylori*.

It is also realized that *Helicobacter pylori* contains large polylactosamine oligosaccharides on its surface which at least in some strains contains non-fucosylated epitopes which can be bound by the bacterium as described by the

20 invention. The substance described herein can also prevent the binding between *Helicobacter pylori* bacteria and that way inhibit bacteria for example in process of colonization.

According to the invention it is possible to incorporate the *Helicobacter pylori* binding substance, optionally with a carrier, in a pharmaceutical composition, which is suitable for the treatment of a condition due to the presence of *Helicobacter pylori* in a patient or to use the *Helicobacter pylori* binding substance in a method for treatment of such conditions. Examples of conditions treatable according to the invention are chronic superficial gastritis, gastric ulcer, duodenal ulcer, non-Hodgkin 30 lymphoma in human stomach, gastric adenocarcinoma, and certain pancreatic, skin, liver, or heart diseases, sudden infant death syndrome, autoimmune diseases including autoimmune gastritis and pernicious anaemia and non-steroid anti-inflammatory drug (NSAID) related gastric disease, all, at least partially, caused by the *Helicobacter pylori* infection.

35

The pharmaceutical composition containing the *Helicobacter pylori* binding substance may also comprise other substances, such as an inert vehicle, or pharmaceutically acceptable carriers, preservatives etc, which are well known to

persons skilled in the art. The *Helicobacter pylori* binding substance can be administered together with other drugs such as antibiotics used against *Helicobacter pylori*.

5 The *Helicobacter pylori* binding substance or pharmaceutical composition containing such substance may be administered in any suitable way, although an oral administration is preferred.

10 The term "treatment" used herein relates both to treatment in order to cure or alleviate a disease or a condition, and to treatment in order to prevent the development of a disease or a condition. The treatment may be either performed in a acute or in a chronic way.

15 The term "patient", as used herein, relates to any human or non-human mammal in need of treatment according to the invention.

It is also possible to use the *Helicobacter pylori* binding substance to identify one or more adhesins by screening for proteins or carbohydrates (by carbohydrate-carbohydrate interactions) that bind to the *Helicobacter pylori* binding substance.

20 The carbohydrate binding protein may be a lectin or a carbohydrate binding enzyme. The screening can be done for example by affinity chromatography or affinity cross linking methods (Ilver *et al.*, 1998).

Furthermore, it is possible to use substances specifically binding or inactivating the 25 *Helicobacter pylori* binding substances present on human tissues and thus prevent the binding of *Helicobacter pylori*. Examples of such substances include plant lectins such as *Erythrina cristagalli* and *Erythrina corallodendron* lectins (Teneberg *et al.*, 1994) or polylactosamine binding lectins such as potatolectin or NeuNAc α 3 specific lectins such as *Sambucus nigra* agglutinin. When used in humans, the 30 binding substance should be suitable for such use such as a humanized antibody or a recombinant glycosidase of human origin which is non-immunogenic and capable of cleaving the terminal monosaccharide residue/residues from the *Helicobacter pylori* binding substances. However, in the gastrointestinal tract, many naturally occurring lectins and glycosidases originating for example from food are tolerated.

35 Furthermore, it is possible to use the *Helicobacter pylori* binding substance as part of a nutritional composition including food- and feedstuff. It is preferred to use the *Helicobacter pylori* binding substance as a part of so called functional or functionalized food. The said functional food has a positive effect on the person's or

animal's health by inhibiting or preventing the binding of *Helicobacter pylori* to target cells or tissues. The *Helicobacter pylori* binding substance can be a part of a defined food or functional food composition. The functional food can contain other acceptable food ingredients accepted by authorities such as Food and Drug

- 5 Administration in the USA. The *Helicobacter pylori* binding substance can also be used as a nutritional additive, preferably as a food or a beverage additive to produce a functional food or a functional beverage. The food or food additive can also be produced by having ,e.g., a domestic animal such as a cow or other animal produce the *Helicobacter pylori* binding substance in larger amounts naturally in its milk.
- 10 This can be accomplished by having the animal overexpress suitable glycosyltransferases in its milk. A specific strain or species of a domestic animal can be chosen and bred for larger production of the *Helicobacter pylori* binding substance. The *Helicobacter pylori* binding substance for a nutritional composition or nutritional additive can also be produced by a micro-organisms such as a bacteria
- 15 or a yeast.

It is especially useful to have the *Helicobacter pylori* binding substance as part of a food for an infant, preferably as a part of an infant formula. Many infants are fed by special formulas in replacement of natural human milk. *Helicobacter pylori* is

- 20 especially infective with regard to infants or young children, and considering the diseases it may later cause it is reasonable to prevent the infection. *Helicobacter pylori* is also known to cause sudden infant death syndrome, but the strong antibiotic treatments used to eradicate the bacterium may be especially unsuitable for young children or infants.

- 25 Furthermore, it is possible to use the *Helicobacter pylori* binding substance in the diagnosis of a condition caused by an *Helicobacter pylori* infection. Diagnostic uses also include the use of the *Helicobacter pylori* binding substance for typing of *Helicobacter pylori*. When the substance is used for diagnosis or typing, it may be
- 30 included in, e.g., a probe or a test stick, optionally constituting a part of a test kit. When this probe or test stick is brought into contact with a sample containing *Helicobacter pylori*, the bacteria will bind the probe or test stick and can be thus removed from the sample and further analyzed.

- 35 Terminal residues include preferably β 3-linked glucuronic acid and more preferably 6-amides such as methylamide thereof. Therefore analogs and derivatives of the sequence can be produced by changing or derivatising the terminal 6-position of the trisaccharide epitopes.

Preferred *Helicobacter pylori* binding substances

The oligosaccharide sequences according to the invention were found to be

5 unexpectedly effective binders when presented on thin layer surface. This method allows polyvalent presentation of the glycolipid sequences. The surprisingly high activity of the polyvalent presentation of the oligosaccharide sequences makes polyvalency a preferred way to represent the oligosaccharide sequences of the invention.

10 The glycolipid structures are naturally presented in a polyvalent form on cellular membranes. This type of representation can be mimicked by the solid phase assay described below or by making liposomes of glycolipids or neoglycolipids.

15 The present novel neoglycolipids produced by reductive amination of hydrophobic hexadecylaniline were able to provide effective presentation of the oligosaccharides. Most previously known neoglycolipid conjugates used for binding of bacteria have contained a negatively charged groups such as phosphor ester of phosphadityl ethanolamine neoglycolipids. Problems of such compounds are negative charge of

20 the substance and natural biological binding involving the phospholipid structure. Negatively charged molecules are known to be involved in numerous non-specific bindings with proteins and other biological substances. Moreover, many of these structures are labile and can be enzymatically or chemically degraded. The present invention is directed to the non-acidic conjugates of oligosaccharide sequences

25 meaning that the oligosaccharide sequences are linked to non-acidic chemical structures. Preferably, the non-acidic conjugates are neutral meaning that the oligosaccharide sequences are linked to neutral, non-charged, chemical structures. The preferred conjugates according to the invention are polyvalent substances.

30 In the previous art bioactive oligosaccharide sequences are often linked to carrier structures by reducing a part of the receptor active oligosaccharide structure. Hydrophobic spacers containing alkyl chains (-CH₂)_n and/or benzyl rings have been used. However, hydrophobic structures are in general known to be involved in non-specific interactions with proteins and other bioactive molecules.

35 The neoglycolipid data of the examples below show that under the experimental conditions used in the assay the hexadecylaniline parts of the neoglycolipid compounds do not cause non-specific binding for the studied bacterium. In the neoglycolipids the hexadecylaniline part of the conjugate forms probably a lipid

layer like structure and is not available for the binding. The invention shows that reducing a monosaccharide residue belonging to the binding epitope may destroy the binding. It was further realized that a reduced monosaccharide can be used as a hydrophilic spacer to link a receptor epitope and a polyvalent presentation structure.

- 5 According to the invention it is preferred to link the bioactive oligosaccharide via a hydrophilic spacer to a polyvalent or multivalent carrier molecule to form a polyvalent or oligovalent/multivalent structure. All polyvalent (comprising more than 10 oligosaccharide residues) and oligovalent/multivalent structures (comprising 2-10 oligosaccharide residues) are referred here as polyvalent structures, though
- 10 depending on the application oligovalent/multivalent constructs can be more preferred than larger polyvalent structures. The hydrophilic spacer group comprises preferably at least one hydroxyl group. More preferably the spacer comprises at least two hydroxyl groups and most preferably the spacer comprises at least three hydroxyl groups.

15

- According to the invention the hydrophilic spacer group is preferably a flexible chain comprising one or several $-CH_2OH-$ groups and/or an amide side chain such as an acetamido $-NHCOCH_3$ or an alkylamido. The hydroxyl groups and/or the acetamido group also protects the spacer from enzymatic hydrolysis in vivo. The
- 20 term flexible means that the spacer comprises flexible bonds and do not form a ring structure without flexibility. A reduced monosaccharide residues such as ones formed by reductive amination in the present invention are examples of flexible hydrophilic spacers. The flexible hydrophilic spacer is optimal for avoiding non-specific binding of neoglycolipid or polyvalent conjugates. This is essential optimal
- 25 activity in bioassays and for bioactivity of pharmaceuticals or functional foods, for example.

A general formula for a conjugate with a flexible hydrophilic linker has the following Formula IV:

30



- wherein L_1 and L_2 are linking groups comprising independently oxygen, nitrogen, sulphur or carbon linkage atom or two linking atoms of the group forming linkages
- 35 such as $-O-$, $-S-$, $-CH_2-$, $-N-$, $-N(COCH_3)-$, amide groups $-CO-NH-$ or $-NH-CO-$ or $-N-N-$ (hydrazine derivative) or amino oxy-linkages $-O-N-$ and $-N-O-$. L_1 is linkage from carbon 1 of the reducing end monosaccharide of X or when $n = 0$, L_1 replaces $-O-$ and links directly from the reducing end C1 of OS.

p₁, p₂, p₃, and p₄ are independently integers from 0-7, with the proviso that at least one of p₁, p₂, p₃, and p₄ is at least 1. CH₁₋₂OH in the branching term {CH₁₋₂OH}_{p₁} means that the chain terminating group is CH₂OH and when the p₁ is more than 1 there is secondary alcohol groups -CHOH- linking the terminating group to the rest of the spacer. R is preferably acetyl group (-COCH₃) or R is an alternative linkage to Z and then L₂ is one or two atom chain terminating group, in another embodiment R is an analog forming group comprising C₁₋₄ acyl group (preferably hydrophilic such as hydroxy alkyl) comprising amido structure or H or C₁₋₄ alkyl forming an amine. And m > 1 and Z is polyvalent carrier. OS is *Helicobacter pylori* binding oligosaccharide sequence and X is a saccharide residue, which can be replaced by carbon (-C-), nitrogen (-N-) or sulphur (-S-) linkage.

Preferred polyvalent structures comprising a flexible hydrophilic spacer according to formula IV include *Helicobacter pylori* binding oligosaccharide sequence(OS) β1-3 linked to Galβ4Glc(red)-Z, and structures OSβ6GlcNAc(red)-Z and OSβ6GalNAc(red)-Z., where "(red)" means the amine linkage structure formed by reductive amination from the reducing end monosaccharides and an amine group of the polyvalent carrier Z.

20 In the present invention the oligosaccharide group is preferably linked in a polyvalent or an oligovalent form to a carrier which is not a protein or peptide to avoid antigenicity and possible allergic reactions, preferably the backbone is a natural non-antigenic polysaccharide.

25 *Ex vivo* uses of the present invention

It is realized that the present invention can be used for inhibition of pathogens especially diarrhea causing *E. coli* *ex vivo* and such method have use in disinfection and preservation type applications. It is preferred to use the receptor oligosaccharide sequences according to the present invention as part of single substances or as single substances or more preferably as composition comprising at least two receptor oligosaccharide sequences from different groups according to the present invention for inhibition pathogens, preferably *E. coli* *ex vivo*. Polyvalent conjugates according to the present invention especially soluble polyvalent conjugates which can agglutinate pathogens, preferably diarrheagenic *E. coli*, are preferred for *ex vivo* uses. One special *ex vivo* embodiment of the invention is the cleansing or disinfection of surfaces, e.g., of tables, medical devices and packages, in hospital or hospital-like environment with a cleanser or disinfectant containing the receptor oligosaccharide sequences described in the present invention. The receptor saccharides described by the invention can also be used as ingredients in a soap or

detergent used for washing or bathing of patients in hospital or hospital-like environment.

Oral infections and oral health products

- 5 It is realized that infections targeted by the present invention spread through oral route, possibly also from nose to the oral cavity. The present invention is directed to the prevention of the infections already in human mouth. The present invention is directed to the treatment of oral infections by at least two different oligosaccharide sequences which can inhibit at least two different binding specificities of pathogen,
- 10 preferably orally infecting bacterium and more preferably a diarrhea causing bacterium. It is preferred to use the receptor oligosaccharide sequences according to the present invention as part of single substances or as single substances or as composition comprising at least two receptor oligosaccharide sequences from different groups according to the present invention for inhibition of oral or nasal
- 15 infections. According to the present invention the receptor oligosaccharide sequences according to the present invention are used as compositions or as separate substances in products inhibiting pathogens, called here mouth hygiene products, in human mouth.
- 20 It is realized that human mouth comprises similar receptors as human intestine especially on proteins at least neolacto-receptors, mannose receptors and oligosaccharide receptors resembling fucose receptors according to the present invention. As a separate embodiment it is realized that the substances and compositions according to the present invention are also useful in inhibiting
- 25 pathogens causing caries. In a specific embodiment the present invention is also directed to the compositions according to the present invention for treatment of other orally spreading infections such as infection causing otitis media or lung infections including influenza, bronchitis or pneumonia. The mouth hygiene products according to the present invention can also be directed against caries, otitis media,
- 30 bronchitis and pneumonia. In a specific embodiment the composition to be used in mouth hygiene product or for inhibition of a pathogen infecting orally comprises at least oligosaccharide sequences Neu5Ac α 3Gal β 4GlcNAc and/or Neu5Ac α 3Gal β 4Glc or more preferably Neu5Ac α 6Gal β 4GlcNAc and/or Neu5Ac α 6Gal β 4Glc and it is directed at least against human influenza virus, preferably for prophylaxis of influenza virus.
- 35

The present invention is especially directed to mouth hygiene products comprising substances or compositions comprising pathogen inhibiting oligosaccharide sequences, especially oligosaccharide sequences according to the invention. The

mouth hygiene product is preferably selected from the group consisting of tooth pastes, mouth wash solutions, mouth tablets, chewing tablets, and chewing gums. It is preferred to use either monovalent receptor oligosaccharide sequences or polyvalent receptor oligosaccharide sequences. In another preferred embodiment the

5 mouth hygiene product comprises polyvalent oligosaccharide sequences according to the present invention. Due to size of human mouth and volume of liquid saliva on its surface relatively small amount of oligosaccharides is enough to obtain saturating rating concentrations of pathogen inhibiting receptors in mouth. The typical amounts of receptor active monovalent epitopes varies from about 100 nmol to 100 µmol of
10 the receptor active oligosaccharide, (at molecular weight 1000 Da this would be 100 µg to 100 mg). More generally useful amounts are estimated to be between about 1-10 µmol. In a separate embodiment the present invention about therapeutical composition is also directed to pathogen inhibiting nasal sprays. The nasal sprays can be directed against otitis media or lung infections.

15

Topical, washing and cosmetic products

It is realized that the common pathogens can spread on human surfaces such as human skin, genital epithelia, hair, household surfaces, and other surfaces in human environment. The oligosaccharide sequences according to the present invention are

20 also useful for prevention of the pathogens also in these environments. It is therefore also preferred to use the oligosaccharide sequences according to the present invention as single substances, as part of single substances, or as composition comprising at least two receptor oligosaccharide sequences from different groups according to the present invention in topical or cosmetic products, for example as
25 creams, lotions, or gels. It is also preferred to use the substances or compositions according to the present invention products aimed for washing human skin, hair or genital epithelia, (which can be also called as personal hygiene products), or for household surfaces, dishes or clothes. Traditional antibiotics have been aimed for use of household washing solutions, but are not useful because of resistance
30 problems which are not likely with the substances according to the present invention. In preferred embodiment polyvalent oligosaccharide sequences are used for washing solutions, in another preferred embodiment monovalent oligosaccharide sequences are used for washing solutions.

35 *Food safety products to be applied to foods or feeds, beverages, drinks and water*
Besides the therapeutic uses in humans or in animals the invention is also directed to the use of receptors and compositions according to the invention for the prevention of the infections by using the invention to neutralize pathogens or bacteria inside or on surfaces of food products. Carbohydrates according to the

present invention can for example be applied on the surfaces of meat products or animal bodies, body parts in meat production to prevent the spreading of pathogens. Use of soluble and other polyvalent conjugates to cover and agglutinate the bacteria are preferred. A specific method to be used on a surface of a solid or semi-solid food product involves contacting the bacteria with the carbohydrates receptors described by the invention and optionally washing away the pathogen carbohydrate complexes. This kind of method is not acceptable with traditional antibiotics. The carbohydrates according to the invention can be also applied to liquid food products or concentrates or powder to make these including milk and liquid milk like products, various beverages including juices, soft drinks, sport drinks, alcoholic beverages and the like.

In a specific embodiment the carbohydrate according to the invention in polymeric form is applied to a liquid food product or a beverage product, potential pathogens are agglutinated by the polyvalent conjugate and the agglutinated complex is removed by a method based on size or solubility of the complex. Non-soluble agglutinates can be removed when these precipitate by standard methods like decanting the solution above the precipitate or more usually more effectively by filtration methods.

20 Filtration methods can be used to remove larger agglutinated complexes.

Preferred foods to be treated with carbohydrates according to the invention includes various animal food products, especially meat products and middle products in processing. Many pathogens including diarrhea causing *E. coli* bacteria are transmitted effectively from vegetables, fruits, salads and other plant foods which are not properly washed. The food stuffs needing washing, but not washed properly or washed with contaminated water are especially problematic in developing countries. The present invention is also directed to methods for increasing food safety of plant foods and other foods in need of washing to control the amount of pathogens, especially pathogenic *E. coli* bacteria in the food products. The invention is especially directed to home customer products and products aimed for the food industry to prevent infections from food. The product is preferentially in solid form as powder or pill or in a capsule containing solutions of the receptors according to the invention, which can be applied to food under processing. Such product can be used to prevent diarrheas in developing countries especially diarrheas in children. The food safety product is also directed to the prevention of travellers diarrheas. The food safety products and feed safety products below can be considered as novel safe preservatives.

Filter products to purify beverages and water

Contaminated drinks and water are major cause of gastrointestinal diseases, especially diarrheas.

The receptors according to the present invention can be also used to make filters to

5 purify pathogens, especially bacteria from liquid food and beverages and water, especially water used for drinking and preparing foods. Preferentially at least two receptor structures are used. Methods are known to produce solid phase materials to which carbohydrate sequences are conjugated to be used as filters for example from cellulose or plastics or agarose and similar materials. The filters according to the

10 invention also includes affinity chromatography material known in the art. Methods to remove bound materials from such filters are known and in a specific embodiment the filter is regenerated by removing the contaminant and optionally sterilizing the filter by heat or other sterilizing means.

15 *Feed safety products*

The food safety products described above can be also applied to animal solid and liquid feeds and drinking water of animals. Preferred target animals to be protected includes pet animals, especially cats and dogs and cattle or farm animal such as cows and other domestic ruminants, pigs, sheep, horses, poultry including for example hens, ducks and turkeys, and rabbits.

Water, food and feed safety analytics

Standard analytic and diagnostic methods in combination with the receptor carbohydrates according to the invention can be applied to water, beverages, foods and feeds to measure presence pathogens binding to the receptor carbohydrates. The knowledge of the binding specificities of contaminating pathogens can be applied to design of therapy when patients are infected or to methods for food safety remove or control pathogens as described above.

30 *Other carbohydrate based interactions which can be inhibited according to the invention*

Beside inhibiting different types of adhesin presentations the invention can be also used to inhibit carbohydrate-carbohydrate interactions and carbohydrate-lectin interactions.

35

The carbohydrate compositions and substances comprises of oligosaccharide sequences. The oligosaccharides inhibit one or several pathogens by binding one or several pathogens and/or by binding the receptors of one or several pathogens. Preferentially at least two pathogen inhibiting oligosaccharide sequences are used

and more preferentially at least three pathogen inhibiting oligosaccharide sequences. In other embodiments at least four, five, six , or seven pathogenesis inhibiting oligosaccharide sequences are used.

- 5 In specific therapies one or several of the oligosaccharide sequences are given separately at different time points. This is especially useful when the administration of all the oligosaccharide sequences would have negative effects on the normal flora. The separate administration of the therapeutic compositions can be useful also because of effect of nutritional situation in the gastrointestinal tract could change
- 10 differently the stability of the on the oligosaccharide sequences according to the inventionin the gastrointestinal tract..

Use of the invention together with probiotic bacteria

When the invention is used to inhibit bacterial binding, especially multiple bacterial

- 15 bindings, also some beneficial bacterial bindings can be prevented. The normal bacterial flora has many important functions for example in the human gastrointestinal system. The destruction of the normal bacterial flora is however an even larger problem with use of traditional antibiotics.
- 20 In a separate embodiment at least two pathogen inhibiting oligosaccharides are administered together with a probiotic microbe and/or a prebiotic substance. The probiotic microbe according to the invention represent a non-harmful bacteria with beneficial functions, for example in digestion of food, providing nutrients and vitamins or covering tissue surfaces from pathogenic bacteria. The probiotic bacteria
- 25 comprise preferentially one or several or multitude of normal bacterial flora. In a preferred embodiment the probiotic bacterium comprise one or several types, strains, or species of lactic acid bacteria.

The prebiotic substance is a substance supporting the normal flora or probiotic

- 30 microbe. Preferred prebiotic substances include prebiotic carbohydrates, such as galactose oligosaccharides, xylose oligosaccharide, or fructose oligosaccharides used as prebiotic substances, the prebiotic substances also include polysaccharides and fibers with prebiotic activities such as inulin or modified starches. The present invention is also directed to the use of other polysaccharides which are used in food
- 35 or for nutritional purposes such as chitosan or beta-glucans for example glucan from oats, which are used to reduce cholesterol and fats. In a preferred embodiment one or several pathogen inhibiting carbohydrates are chosen so that they are also prebiotic substances like carbohydrates with a non-reducing terminal beta linked galactose residue In a preferred form of therapy

- a) pathogens and potentially part of the normal flora are first removed by one or more preferentially at least two carbohydrates according to the invention
- b) probiotic microbe and/or prebiotic substance are applied.

Steps 1 and 2 may also be applied in reversed order, preferably with a large amount
 5 of the probiotic microbe and/or prebiotic substance and then step one. According to
 the invention it is also possible to repeat steps 1 and/or 2 several times while varying
 10 the order of the steps. Steps 1 and 2 may be applied at the same time. The substances
 according to the invention can be administered together with probiotic microbe
 and/or prebiotic substance or alternatively probiotic microbe and/or prebiotic
 15 substance can be included in the compositions according to the invention, and then
 steps 1 and 2 above can be performed simultaneously.

Some of the oligosaccharide sequences according to the invention are known to have
 prebiotic effects, these includes N-acetyl-lactosamine type oligosaccharide
 15 sequences, and fucosylated oligosaccharides, especially human milk
 oligosaccharides. Administration human milk oligosaccharides together with a
 probiotic microbe and/or prebiotic substance, especially N-acetyllactosamine
 containing for example one or several from the group Lacto-N-neotetraose, Lacto-N-
 20 tetraose, Lacto-N-hexaose, Lacto-N-neohexaose, para-Lacto-N-hexaose, para-Lacto-
 N-neohexaose, and/or fucosylated oligosaccharides derived from these such as
 and/or mono-di- or trifucosylated Lacto-N-tetraose (LNT) or/or Lacto-N-neotetraose
 (LNnT) and/ or fucosyl-lactose oligosaccharides such as 2'-fucosyl-lactose, and /or 3-
 25 fucosyllactose, and/or difucosyllactose.

25 Other useful substances to be used with the substances and/or compositions
 according to the invention

According to the present invention it is also useful to use the pathogenesis
 preventing carbohydrate together with a glycosidase inhibitor, preferably sialidase
 30 inhibitor.

According to the present invention it is also useful to use the pathogenesis
 preventing carbohydrate together with a lectin or another carbohydrate binding
 protein. The lectin can be used to block carbohydrate receptors, for example on the
 bacterial exopolysaccharides.

35 Glycolipid and carbohydrate nomenclature is according to recommendations by the
 IUPAC-IUB Commission on Biochemical Nomenclature (Carbohydrate Res. 1998,
 312, 167; Carbohydrate Res. 1997, 297, 1; Eur. J. Biochem. 1998, 257, 29).

It is assumed that Gal, Glc, GlcNAc, and Neu5Ac are of the D-configuration, Fuc of the L-configuration, and all the monosaccharide units in the pyranose form.

Glucosamine is referred as GlcN or GlcNH₂ and galactosamine as GalN or GalNH₂. Glycosidic linkages are shown partly in shorter and partly in longer nomenclature,

- 5 the linkages of the Neu5Ac-residues α 3 and α 6 mean the same as α 2-3 and α 2-6, respectively, and with other monosaccharide residues α 1-3, β 1-3, β 1-4, and β 1-6 can be shortened as α 3, β 3, β 4, and β 6, respectively. Lactosamine refers to N-acetyllactosamine, Gal β 4GlcNAc, and sialic acid is N-acetylneuraminic acid (Neu5Ac, NeuNAc or NeuAc) or N-glycolylneuraminic acid (Neu5Gc) or any other
- 10 natural sialic acid. Term glycan means here broadly oligosaccharide or polysaccharide chains present in human or animal glycoconjugates, especially on glycolipids or glycoproteins. In the shorthand nomenclature for fatty acids and bases, the number before the colon refers to the carbon chain length and the number after the colon gives the total number of double bonds in the hydrocarbon chain.
- 15 Abbreviation GSL refers to glycosphingolipid. Abbreviations or short names or symbols of glycosphingolipids are given in the text and in Tables 1 and 2.

Helicobacter pylori refers also to the bacteria similar to *Helicobacter pylori*.

- 20 The expression "terminal oligosaccharide sequence" or "terminal sequence" indicates that the oligosaccharide sequences or sialic acid derivative sequences are not linked to other monosaccharide or oligosaccharide structures except optionally from the reducing end of the oligosaccharide sequence. The oligosaccharide sequence when present as conjugate is preferably conjugated from the reducing end of the oligosaccharide sequence, though other linkage positions which are tolerated
- 25 by the pathogen binding can also be used. In a more specific embodiment the oligosaccharide sequence according to the present invention means the corresponding oligosaccharide residue which is not linked by natural glycosidic linkages to other monosaccharide or oligosaccharide structures. The oligosaccharide residue is preferably a free oligosaccharide or a conjugate or derivative from the
- 30 reducing end of the oligosaccharide residue.

The term " α 3/ β 3" indicates that the adjacent residues in an oligosaccharide sequence can be either α 3- or β 3-linked to each other.

- 35 The present invention is further illustrated by the following examples, which in no way are intended to limit the scope of the invention:

EXPERIMENTAL SECTION

Ganglioside Preparations – For isolation of gangliosides a number of tissues, previously described to contain complex gangliosides, as e.g. human erythrocytes, bovine erythrocytes, rabbit thymus, human meconium and human cancers (Stults et al., 1989), were collected. Isolation of total acid glycosphingolipid fractions was done as described previously (Karlsson 1987). Briefly, the tissues were lyophilised, followed by extraction in two steps with chloroform/methanol (2:1 and 9:1, by volume) in a Soxleth apparatus. The material obtained was pooled, subjected to mild alkaline hydrolysis and dialysis, followed by separation on a silicic acid column. Acid and non-acid glycosphingolipids were separated on a DEAE column.

The acid glycosphingolipid fractions were separated by DEAE-Sepharose chromatography, followed by repeated silicic acid chromatography, and final separation was achieved using HPLC on Kromasil 5 silica column of length 250 mm, inner diameter 10 mm and particle size of 5 µm (Phenomenex, Torrance, CA, USA) using linear gradients of chloroform/methanol/water (60:35:8 to 40:40:12 or 65:25:4 to 40:40:12, by volume) over 180 min, with a flow rate of 2 ml/min. The 2 ml fractions collected were analyzed by thin-layer chromatography and anisaldehyde staining (see below), and the *H. pylori* binding activity was assessed using the chromatogram binding assay (see below). The fractions were pooled according to the mobility on thin-layer chromatograms and their *H. pylori* binding activity.

Reference Glycosphingolipids – Reference glycosphingolipids were isolated and characterized at the Institute of Medical Biochemistry, Göteborg University, Sweden. Structural characterisation was performed using proton NMR (Koerner et al., 1983), mass spectrometry (Samuelsson et al., 1990) and degradation studies (Yang and Hakomori 1971, Stellner, 1973). Sialyl-Le^x hexaglycosylceramide was purchased from ARC, Edmonton, Canada.

Bacterial Strains, Growth Conditions and Labeling – *H. pylori* strain CCUG 17874 was obtained from the Culture Collection University of Göteborg (CCUG. Strain J99 was kindly provided by Drs. Tim Cover, John Atherton and Martin Blaser.

Bacteria were grown on Brucella medium (Difco Laboratories, Irvine, CA) containing 10% fetal calf serum (Harlan Sera-Lab Loughborough, UK) inactivated at 56 °C, and BBL IsoVitale X Enrichment (Becton Dickson Microbiology Systems, Franklin Lakes, NJ). Bacteria were radiolabelled by the addition of 50 µCi ³⁵S-methionine (Amersham Pharmacia Biotech, Little Chalfont, U.K) diluted in 0.5 ml

phosphate-buffered saline (PBS) pH 7.3, to the culture plates. After incubation for 12-72 h at 37 °C under microaerophilic conditions, the bacteria were harvested and centrifuged three times at 3,500 rpm for 10 min in PBS.

5 Alternatively, colonies were inoculated (1×10^5 CFU/ml) in Ham's F12 medium (Invitrogen Corp., Carlsbad, CA, UK), supplemented with 10% heat-inactivated fetal calf serum and 50 µCi ^{35}S -methionine. The culture bottles were incubated with shaking under microaerophilic conditions at 37 °C for 24 h. Bacterial cells were harvested by centrifugation, and washed three times with PBS.

10 In both cases, the bacteria were finally resuspended in PBS containing 2% (w/v) bovine serum albumin (PBS/BSA) to approximately 1×10^8 CFU/ml. Both labeling procedures resulted in suspensions with specific activities of approximately 1 cpm per 100 *H. pylori* organisms.

15 *Thin-Layer Chromatography* – Total acid glycosphingolipid fractions (40 µg) or pure gangliosides (0.0002-4 µg) were separated on aluminium backed silica gel 60 HPTLC plates (Merck, Darmstadt, Germany) using chloroform/methanol/water (60:35:8, by volume), chloroform/metanol/0.25% KCl in water (50:40:10, by volume) or 1-propanol/water/25% NH₃ (7:3:1, by volume) as the solvent systems.

20 Chemical detection of glycosphingolipids on thin-layer chromatograms was carried out using anisaldehyde (Waldi 1962) or resorcinol (Svennerholm 1963) reagents.

25 *Chromatogram Binding Assay* – The chromatogram binding assay was essentially carried out as described previously (Ångström et al., 1994). Dried thin-layer chromatograms with separated glycosphingolipids were treated in 0.5% polyisobutylmethacrylate (w/v) (Aldrich Chemical Company Inc., Milwaukee, WI) in diethylether/*n*-hexane (1:5, by volume) for 1 min and then air dried. To reduce non-specific binding plates were incubated in PBS/BSA containing 0.1% NaN₃ (w/v) and 0.1% Tween 20 (by volume) at room temperture for 2 h. The plates were then incubated for 2 h at room temperture with ^{35}S -labeled *H. pylori* diluted in PBS/BSA. Binding of ^{125}I -labeled cholera toxin B-subunits to dilutions of the GM1 ganglioside on thin-layer chromatograms was done as described (Karlsson and Strömborg, 1987). Following the final wash and drying, autoradiography was carried

30 out over night using Biomax film (Eastman Kodak Company, NY, USA). To assess the quantity of bacterial binding to one pure ganglioside relative to others, binding of bacteria to ganglioside dilutions on thin-layer chromatograms and autoradiography was followed by densitometry of the autoradiographs. The range of ganglioside concentrations were in each case chosen on the basis of pilot experiments where the

concentrations giving saturation were determined. The autoradiographs were replicated using a CCD camera (Dage-MTI, Inc., Michigan City, In), and analysis of the images was performed using the public domain NIH Image program (developed at the U.S. National Institutes of Health, and available at <http://rsb.info.nih.gov/nih-image>). Data are presented after subtraction of background values.

Derivatisations of Gangliosides – Gangliosides were permethylated using sodium hydroxide and methyl iodide in dimethyl sulphoxide as described previously described (Larsson et al., 1987). Reduction of permethylated samples was carried out 10 using LiAlH₄ in diethylether (Karlsson 1974).

Fast Atom Bombardment and Electron Ionisation Mass Spectroscopy – Negative ion FAB and EI mass spectra were obtained on a JEOL SX 102A mass spectrometer (JEOL, Toyko, Japan). Negative ion FAB mass spectra of native gangliosides were 15 obtained using Xe atom bombardment (6eV), an acceleration voltage of -8kV and triethanolamine as matrix. EI spectra of derivatized glycosphingolipids were obtained with an ionisation voltage of 70 eV, an ionsiation current of 300 μA and an acceleration voltage of 8 kV. The temperature was raised from 150 °C to 410 °C at a rate of 10 °C/min. For the collection of both FAB and EI spectra a resolution of 1000 20 was used.

Electrospray Ionisation Mass Spectrometry and Collision Induced Dissociation – The permethylated *H. pylori*-binding ganglioside of human erythrocytes was dissolved in 10% acetonitrile in water (by volume). Electrospray ionisation was 25 carried out on a Q-TOF mass spectrometer (Micromass, UK) using the nanoflow electrospray option with a static flow rate of approximately 15-40 nL/min. A spray was generated by the application of 900V across the nanospray needle. Source temperature was 80 °C. Nitrogen at a flow rate of 150 l/h was used as drying gas. The doubly charged peak at *m/z* 1594.4 observed in the mass spectrum was chosen for 30 collision induced dissociation. Collision induced dissociation was achieved using argon with a collision energy of 30-80V. Data were collected over a mass range of 50-2600 mass units. The nomenclature of Domon and Costello (Domon and Costello, 1988) was used in this context.

35 Proton NMR Spectroscopy – ¹H NMR spectra were acquired on Varian 500 MHz and 600 MHz spectrometers at 30 °C. The samples were dissolved in dimethyl sulphoxide/D₂O (98:2, by volume) after deuterium exchange.

Source of natural glycolipids

PGCs (human erythrocytes) were isolated by us according to the peracetylation method (Miller-Podraza et al. 1993). S-3-PG (human erythrocytes and human leukocytes), disialylparagloboside (human erythrocytes), S-6-PG (human leukocytes), 7-sugar neolacto ganglioside (human erythrocytes and leukocytes) and globoside (human erythrocytes) also were prepared in our laboratory (Karlsson 1987). GQ1b of human brain was from Department of Neurochemistry of Göteborg University (Miller-Podraza et al. 1992). Gangliosides GM1, GD1a, GD1b and GT1b of bovine brain were purchased from Calbiochem (USA).

Source of carbohydrates

NeuAc α 3Gal β 4GlcNAc β 3Gal β 4Glc was prepared in our laboratory from S-3-PG (human erythrocytes) using ceramide glycanase (from leech, Boehringer Mannheim 15 GmBH, Germany) digestion (Ito and Yamagata 1989) and phase partition in chloroform/methanol/water, 2:1:0.6. The pentasaccharide was recovered from the upper phase. NeuAc α 3Gal β 3GlcNAc β 3Gal β 4Glc, NeuAc α 6Gal β 4GlcNAc β 3Gal β 4Glc and Gal β 3(NeuAc α 6)GlcNAc β 3Gal β 4Glc were from IsoSep (Tullinge, Sweden)

20

Other reagents

4-Hexadecylaniline, methylamine, ethylamine, propylamine, butylamine and benzylamine were from Aldrich Chemical Company (Milwaukee, USA). Glycolic acid, ethanolamine and octadecylamine were purchased from Sigma-Aldrich 25 (Germany). Sephadex LH 20 was from Pharmacia (Uppsala, Sweden) and ethylene glycol from Fluka (Sweden).

Chemical modifications of the sialic acid glycerol tail

30 *A. Mild periodate oxidation* (Veh et al. 1977) followed by reduction (R-CHOH-CHOH-CH₂OH -> R-CHOH-CH₂OH/R-CH₂OH):
The material (0.5-1 μ mol) was incubated in 500 μ l of 0.05 mM acetate buffer, pH 5.5, containing 1-2 mM NaIO₄, for 40 min on ice. The reaction was terminated with an excess of ethylene glycol. The sample was then concentrated by freeze drying 35 (about 5-fold) and reduced with an excess of NaBH₄ at room temperature, overnight. Finally the sample was dialyzed against distilled water for 2 days and freeze dried.
B. Mild periodate oxidation of S-3-PG followed by coupling with methylamine or ethanolamine (R-CHOH-CHOH-CH₂OH -> R-CH₂-NH-CH₃/R-CH₂-NH-CH₂-OH). After oxidation of S-3-PG with mild periodate (see above) and addition of ethylene

glycol, the material was dialyzed for 2 days against distilled water and freeze dried. The oxidized S-3-PG was coupled with methylamine or ethylamine under the following conditions: the glycolipid (0.5 mg) was dissolved in 200 µl of M/C 3:1 and mixed with 50 µl of amine, 200 µl of NaBH₃CN in methanol (62 mg/ml) and 5 200 µl of glycolic acid in water (136 mg/ml). In the case of methylamine, 50 µl of tetrahydrofuran was added to improve solubility. The sample was incubated at 30°C for 4 h and evaporated under nitrogen. The residue was suspended in C/M/water, 60:30:4.5, and desalted using Sephadex LH-20 column packed in methanol. After application of the sample (about 0.6 ml per 0.5x15 cm column), the column was 10 eluted with methanol and the glycolipid was recovered by collecting sugar-positive fractions (monitored by TLC and anisaldehyde). For final purification of molecular species (see formulae above), the material was separated by preparative TLC using C/M/water, 60:35:8, as developing system. The main band (detected with anisaldehyde after cutting off a strip from the plate) was scraped out and extracted 15 with the same solvent.

Modifications of the carboxyl group (Lanne et al.1995)

S-3-PG (0.5-5 mg) was first converted to the methylester (R-COOH → R-COCH₃) by incubation with methyl iodide (100 µl) in dimethylsulfoxide (DMSO, 0.5 ml), for 20 1h at room temperature. The product was purified using Sephadex LH-20, as described above. To prepare the alcohol derivative (R-COCH₃ → R-CH₂OH) of S-3-PG, the methylester (0.5 mg) was dissolved in 0.5 ml of methanol, followed by addition of 5 mg of NaBH₄. After 1 h at room temperature the reduced S-3-PG was desalted using Sephadex LH-20 (see above).

25 For synthesis of the amide and the methyl-, ethyl-, propyl-, benzyl- and stearyl amide of S-3-PG, the methyl ester (0.5 mg in 0.5 ml of methanol) was mixed with: 0.2 ml of 30% NH₃ in water, 0.5 ml of 40% methylamine in water, 1 ml of 70% ethylamine in water, 100 µl of propylamine, 100 µl of benzylamine or 200 µl 30 stearylamine in THF (saturated solution), respectively. After incubation at room temperature overnight, the products were evaporated under nitrogen. Benzyl and stearyl amides were further purified by extraction with hexane/acetone 1:1 (by vol.). The samples were washed with excess of the above solvent mixture, centrifuged and the supernatants were discarded.

35 PGC derivatives were prepared in the same way with the following exceptions. 1, the reduced PGC preparation was separated on DEAE-Sephadex column and only the neutral fraction was further investigated and 2, the oxidation/reduction procedure was performed twice.

Coupling of hexadecylaniline to free saccharides (30)

Saccharide (0.5 mg) was dissolved in 100 µl of methanol and mixed with 100 µl of NaBH₃CN in methanol (62 mg/ml), 100 µl of hexadecylaniline in tetrahydrofuran

5 (40 mg/ml) and 100 µl of 1.8 M glycolic acid in water (136 mg/ml). Additional 100-200 µl of tetrahydrofuran were added to improve solubility of precipitating hexadecylaniline. The sample was incubated at 30° C overnight and the product purified by Sephadex LH chromatography (see "modifications of the carboxyl group" above). The yield of this reaction was more than 90%.

10

Synthesis of branched lipid parts (Magnusson et al. 1994, Read et al. 1977)

In order to obtain neoglycolipids with branched lipid parts the HDA-derivatized saccharides were further modified by *N*-acylation. 500 µl of *p*-nitrophenylpalmitate in dry DMSO was added to 200-300 µg of the dried HDA-saccharide. Four drops of

15 triethylamine were added to the sample which was incubated in nitrogen atmosphere at 37° C for 3 days. Glycolipids were purified using Sephadex LH-20 column chromatography (see above) and preparative TLC. The yield was about 20%.

Preparation of lactones

20 S-3PG was transformed into its lactone form by the method described by Laferrière and Roy (33). 1 mg of S-3PG (1 mg) was dissolved in concentrated acetic acid, and the reaction was allowed to proceed at room temperature for two days. The acetic acid was then evaporated and the remaining material dissolved in C/M/water, 60:35:8 (0.5 ml), by volume. The yield of the reaction was controlled by thin-layer chromatography on aluminium-backed HPTLC plates coated with 0.1 mm silica gel 25 60 (Merck, Germany) and was about 50%.

Synthesis and preparation of ganglioside GM1b

Gangliotetraosylceramide, prepared by desialylation of the ganglioside GM1, was 30 sialylated at the terminal galactose using an α -2,3-sialyltransferase (EC number 2.4.99.4). The conditions were as described before (Lee et al. 1994) with slight modifications, as follows. Gangliotetraosylceramide (100 µg) was dissolved in 10 µl of 500 mM MES (4-morpholineethane-sulfonic acid) buffer, pH 6.0, containing Triton CF-54 (2%) and diluted with 74.1 µl water. Then, 10 µl of CMP-NeuAc (15 mM in water) and 6 µl of α -2,3-sialyltransferase (338 mU/ml) were added to the 35 reaction mixture and the sample was left at room temperature over night. The progress of the reaction was analysed by TLC using C/M/0.25% KCl_{aq} (50:40:10, by volume) as eluent. The reaction mixture was then evaporated under a stream of nitrogen, dissolved in C/M/H₂O, (60:30:4.5, by volume, (2 ml) and applied to a

small column (ca 2.5x0.6 cm) packed with Sephadex G-25 (prewashed with 5 ml of the same solvent system). The glycolipid material was eluted with 2.5 ml of the above solvent mixture and 2.5 ml C/M, (2:1, by vol.). Finally, the material was evaporated and dissolved in a small volume of the C/M/H₂O, (60:30:4.5).

5

Helicobacter pylori strains

H. pylori strain CCGU 17874 was from Culture Collection Göteborg University, Sweden, and *H. pylori* strain 032 was a gift from Prof. T. Wadström from Department of Medical Microbiology, Lund University, Sweden. The conditions of bacterial growth in Ham's F12 liquid medium and on *Brucella* agar plates, respectively, were as described previously (Miller-Podraza et al. 1996). In this paper we used the strain CCGU 17874 from agar plates for expressing the binding to S-3-PG (linear structure) and the strain 032 from liquid medium for expressing binding to PGCs (branched structures).

15

RESULTS

Gangliosides Preparations – To be able to dissect the ganglioside binding preferences of *H. pylori* a ganglioside library was compiled (summarized in Table I). Each ganglioside was characterized by mass spectrometry and proton NMR. The procedure is illustrated by the following description of the isolation and characterization of one *H. pylori*-binding ganglioside of human erythrocytes (No. 19 in Table I).

Total acid glycosphingolipids were isolated from 500 l of pooled blood group B erythrocytes by standard procedures (Karlsson 1987), yielding 5.5 g. A sub-fraction of 390 mg were separated on a 700 ml DEAE-Sepharose column eluted with a linear gradient using 2100 ml of ammonium acetate in methanol (0.05 M to 0.45 M). Each 10 ml fraction collected was analyzed by thin-layer chromatography using the resorcinol reagent. The fractions were pooled according to the mobility of the major compounds. Pooling of fractions 62-67 yielded 15.4 mg, and the fraction obtained had a major compound migrating in the sialyl-neolactotetraosylceramide region. However, when tested for *H. pylori*-binding activity using the chromatogram binding assay, a slow-migrating binding-active compound was detected. The 15.4 mg of acid glycosphingolipids were further separated by HPLC using a linear gradient of chloroform/methanol/water (60:35:8 to 40:40:12, by volume). The *H. pylori*-binding compound eluted in fractions 39-56, which after pooling yielded 0.9 mg.

The characterization of the glycolipid structure is shown in Figures 1-5 and will be published more in detail elsewhere.

Binding of H. pylori to the Ganglioside Library

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The *H. pylori* strains CCUG 17874 and J99, used in the chromatogram binding experiments, are both sialic acid binding (Mahdavi et al., 2002).

I. Binding and Non-Binding Gangliosides – The results from binding of the *H. pylori* strains to the isolated gangliosides are exemplified in Figs. 6-10, and summarized in Table I. Gangliosides were classified as non-binding when no binding was obtained although 4 µg of the compound was applied on the thin-layer plates. As shown in Fig. 6 the sialic acid-binding wild type strains CCUG 17874 and J99 recognized the NeuAc-terminated gangliosides

10 NeuAc α 3Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer (NeuAc-neolactohexaacylceramide; lane 1), NeuAc α 3Gal β 4GlcNAc β 6 (NeuAc α 3Gal β 4GlcNAc β 3)Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer (NeuAc-G-10 ganglioside; lane 3), Gal α 3(Fuc α 2)Gal β 4GlcNAc β 6 (NeuAc α 3Gal β 4GlcNAc β 3)Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer (G9-B ganglioside; lane 6), but not the corresponding NeuGc-terminated isostructures (lanes 2, 4 and 5). Further gangliosides recognized were NeuAc α 3(Gal β 4GlcNAc β 3) $_3$ Gal β 4Glc β 1Cer (NeuAc-neolactooctaacylceramide, Fig. 8, lanes 1-7), NeuAc α 3Gal β 4GlcNAc β 3Gal β 4(Fuc α 3)GlcNAc β 3Gal β 4Glc β 1Cer (VIM-2 ganglioside, Fig. 9, lanes 6-10), and

15 NeuAc α 3Gal β 4(Fuc α 3)GlcNAc β 3Gal β 4(Fuc α 3)GlcNAc β 3Gal β 4Glc β 1Cer (sialyl-dimeric-Le^x ganglioside, Fig. 9, lanes 11-14).

An occasional binding to NeuAc α 3-neolactotetraosylceramide (No. 2 in Table I; Fig. 8, lanes 1-7) was also detected, while NeuAc α 6-neolactotetraosylceramide (No. 30 3) was non-binding, in line with previous reports (Miller-Podraza et al, 1997, Johansson and Miller-Podraza 1998). The NeuAc α 6-carrying gangliosides Gal β 4GlcNAc β 6(NeuAc α 6Gal β 4GlcNAc β 3)Gal β 4Glc β 1Cer (No. 14) and Gal β 4GlcNAc β 6(NeuAc α 6Gal β 4GlcNAc β 3)Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer (No. 35 15) were also non-binding. No binding to NeuGc α 3-neolactotetraosylceramide (No. 4) or disialyl-neolactotetraosylceramide (No. 5) was obtained. An occasional binding to the sialyl-Le^x hexaglycosylceramide (No. 7) was observed. The sialyl-Le^a hexaglycosylceramide (No. 6) was not recognized by the CCUG 17874 strain, while the J99 strain occasionally bound to this compound.

II. Comparison of Relative Binding Affinities – Binding of radiolabeled *H. pylori* to glycosphingolipids diluted in microtiter wells was initially attempted in order to appreciate the relative binding affinities for the various binding-active gangliosides. However, the results thereby obtained were not reproducible. Therefore, binding assays using dilutions of gangliosides on thin-layer plates were utilized. In initial experiments we found that the binding to NeuAc α 3-neolactohexaosylceramide and the NeuAc-dimeric-Le x ganglioside was saturated at approximately 100 pmole, and therefore lower concentration ranges (1-100 pmole) were utilized in order to obtain binding curves. The results presented in Figs. 8-10 are representative of a large number of binding assays. Although the level of binding varied somewhat between different batches of radiolabeled bacteria, the same relationships between the binding-active gangliosides were repeatedly obtained. To allow a comparison with a well characterized ganglioside recognition system, the result from binding of 125 I-labeled cholera toxin B-subunits to dilutions of the GM1 ganglioside on a thin-layer chromatogram is included in Fig. 10.

A. Effect of Carbohydrate Chain Length – Binding of *H. pylori* strain CCUG 17874 to dilution series of NeuAc α 3-neolactotetraosylceramide (No. 2 in Table I), NeuAc α 3-neolactohexaosylceramide (No. 8), and NeuAc α 3-neolactooctaosylceramide (No. 10), demonstrated a clear preference for NeuAc α 3-neolactooctaosylceramide (Fig. 8).

B. Effect of Branching – To evaluate the effect of branching of the carbohydrate chain, the binding of *H. pylori* strain CCUG 17874 to NeuAc α 3-neolactohexaosylceramide (No. 8), the NeuAc-G-10 ganglioside (No. 16) and the G9-B ganglioside (No. 19), Table 1, was compared. As shown in Fig. 9 the blood group B type 2 epitope on the β 6-linked branch of the G9-B ganglioside impaired the binding when compared to the linear NeuAc α 3-neolactohexaosylceramide. On the other hand, since the NeuAc-G-10 ganglioside was the preferred ligand, it would indicate that the NeuAc α 3Gal β 4GlcNAc sequence on the β 6-linked branch in this case increased the binding affinity.

C. Effect of Fucose Residues – To investigate the effect of fucose branches the relative binding of *H. pylori* strain CCUG 17874 to NeuAc α 3-neolactohexaosylceramide (No. 8), the VIM-2 ganglioside (No. 12) and the sialyl-dimeric-Le x ganglioside (No. 13) was assessed. As shown in Fig. 10 the bacteria bound with higher affinity to the VIM-2 ganglioside and the sialyl-dimeric-Le x ganglioside compared to NeuAc α 3-neolactohexaosylceramide.

Detailed epitope dissection of the terminal NeuNAc α 3LacNAc-structures

1. Binding epitope associated with linear carbohydrate chains

S-3-PG

5 S-3-PG, chosen as a model compound for chemical derivatizations, was the simplest ganglioside of human neutrophils which bound *H. pylori* in our overlay assay (Fig. 11). As shown in the figure, human neutrophils contain a mixture of gangliosides with binding affinity for the bacterium and the strength of binding appears to be higher for more complex, slower migrating species. The minimum amount of S-3PG required for a positive reaction in a typical experiment on TLC plates was 20-30 pmols per 1 x 7 mm spot, which corresponds to 2.8-4.2 pmol/mm². For some *H. pylori* batches the sensitivity of S-3-PG detection was even higher reaching the level of 0.3-0.4 pmol/mm².

10 15 *Chemically modified S-3-PG*

S-3-PG was chemically modified in different ways in order to test the importance of the glycerol tail and the carboxyl group of NeuAc in the interaction with *H. pylori*. The following derivatizations were performed: (a) mild periodate oxidation of the sialic acid glycerol tail followed by reduction or mild periodate oxidation followed by coupling with methylamine or ethanolamine, (b) reduction of the carboxyl group to primary alcohol, (c) conversion of the carboxyl group to various amides, (d) synthesis of lactones. The derivatives were investigated by negative ion FAB MS in order to confirm the identity of the structures, see Fig 12. All molecular ions were in agreement with expected masses and the changes were limited to the sialic acid residue (Table 2). The latter was shown by unchanged fragments ions indicating sequence of sugars in the core chain at *m/z* 1339, 1176, 973 and 811 (Y series of ions according to current nomenclature, Domon and Costello 1988 and Harvey 1999). The undestroyed ceramide part was shown by a fragment ion at *m/z* 649 (Y_0 ion) (18:1-24:0). Each of these ions appear together with a satellite ion (-28 mass units) due to the presence of some amounts in the preparation of S-3PG with the d18:1-22:0 ceramide (e.g., *m/z* 1629 and 1601 in Fig 2A or 1582 and 1554 in Fig. 2C). The only exception is Fig. 2B where the ions at *m/z* 1599 and 1569 represent two different derivatives, obtained from the main component 1629 (2A). In Fig. 2B the ions with d:18-22-0 melt together with the background.

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All derivatives obtained from S-3PG were tested for binding by *H. pylori* using overlay of TLC plates with radiolabeled bacteria and the results are summarized in Table 2. An example of binding studies is given in Fig. 13. As shown, most of the

modifications performed on the sialic acid residue eliminated or drastically reduced the binding of *H. pylori* to S-3PG. There was some binding to amide and benzylamide derivatives of S-3PG. Part of the se bindings required higher amounts of the glycolipid material. There was also some interaction with oxidized/reduced

5 SPG on some of the plates (not shown), probably due to the presence of trace amounts non-derivatized SPG in the preparation. To further investigate the importance of the glycerol tail, S-3-PG was oxidized and coupled with methylamine or ethanolamine. The two main derivatives obtained were R-CH₂-NH-CH₃ and R-

10 CH₂-NH-CH₂-CH₂OH. Both turned out to be inactive as binding molecules on TLC plates. In contrast, strong binding was observed for octadecylamide of S-3PG which interacted with *H. pylori* on TLC plates at a lower pmol level (Fig. 14).

Other glycolipids and neoglycolipids

A panel of different natural gangliosides and neogangliosides was tested for interaction with *H. pylori* (Table 3). There was neither binding of the bacterium to gangliosides of the ganglio series, nor to S-6-PG, NeuAc α 8NeuAc α 3-PG, or neogangliosides prepared from sialylated oligosaccharides based on the lacto (Gal β 3GlcNAc β 3Gal β 4Glc) core chain. On the other hand, the S-3PG saccharide coupled with HDA or with a branched lipid chain was active under the same overlay conditions. Binding of *H. pylori* to neoglycolipids synthesized in our lab are shown in Fig. 15. The structures of the main bands were confirmed by mass spectrometry after scraping off the material from the plate. The arrows in lane 2 indicate bands corresponding to the HDA derivative (lower band) and the branched derivative (upper double band). FAB $^-$ spectra of these fractions are shown in Fig. 16. The molecular ions and fragment ions were as expected. Similar FAB $^-$ spectra were obtained for corresponding fractions shown in lanes 3-5 of Fig. 15.

25 NeuAc α 3Gal β 4GlcNAc β 3Gal β 4Glc saccharide was obtained from a SPG preparation which contained minor amounts of other sialylated molecules of the binding series (lane 1 in Fig. 15, see also Fig. 11). These minor binding fractions are seen even after derivatization (lane 2 in Fig. 15). Repeated experiments showed that reproducibility of binding of *H. pylori* to HDA neoglycolipids on TLC plates was lower than for derivatives with branched lipid chains.

2. Binding epitope associated with branched poly-N-acetyllactosamine chains of PGCs.

As discussed earlier, this binding could be expressed selectively by some strains of *H. pylori* grown in liquid cultures. As an example, see Fig.18 where there is binding to PGCs but not to S-3PG. The minimum amount of PGCs on TLC plates required for a positive reaction in typical experiments was 0.16 pmol NeuAc/mm². The

glycerol tail of NeuAc of PGCs was modified using mild periodate oxidation followed by reduction ($\text{R-CHOH-CHOH-CH}_2\text{OH} \rightarrow \text{R-CHOH-CH}_2\text{OH/R-CH}_2\text{OH}$) or coupling with ethanolamine ($\text{R-CHOH-CHOH-CH}_2\text{OH} \rightarrow \text{R-CH}_2\text{-NH-CH}_2\text{-OH}$), and the carboxyl group was modified by reduction ($\text{R-COOH} \rightarrow \text{CH}_2\text{OH}$). The

5 modified PGCs were tested by EI MS after permethylation, as exemplified in Fig 17 which shows ions corresponding to terminal sialic acid residue before (6A) and after reduction (6B). As expected, fragments ions at m/z 376 and 344 representing NeuAc were replaced by ions at m/z 362 and 330, corresponding to reduced NeuAc.

10 Binding tests using overlay of TLC plates with radiolabeled bacteria showed that the modifications influenced negatively interaction of PGCs with *H. pylori*. An example of binding of *H. pylori* to modified PGCs is shown in Fig.18 and the results are summarized in Table 4.

15 DISCUSSION

20 Recognition of sialic acid-containing glycoconjugates by certain *H. pylori* strains has been repeatedly demonstrated (Roche et al., 2001, Mahdavi, et al., 2002, Miller-Podraza et al, 1997, Johansson and Miller-Podraza 1998). In the present study a library of gangliosides was collected and used for dissection of *H. pylori* binding preferences utilizing representative sialic acid-recognizing *H. pylori*.

An occasional binding of the J99 strain to sialyl-Le^a hexaglycosylceramide was observed, while the CCUG 17874 strain did not recognize this ganglioside. Binding 25 of the J99 strain to both sialyl-Le^a- and sialyl-Le^x-neoglycoproteins has also been demonstrated (Mahdavi, 2002). This indicates that the SabA carbohydrate binding sites of J99 strain and the CCUG 17874 strain are not identical. However, in all other respects the CCUG 17874 strain and the J99 strain bound to gangliosides in an identical manner, and both strains recognized *N*-acetyllactosamine-based 30 gangliosides with terminal NeuAc α 3, but not NeuAc α 6, in line with previous reports (Miller-Podraza et al, 1997, Johansson and Miller-Podraza 1998). Furthermore, gangliosides with terminal NeuGc α 3 or NeuAc α 8NeuAc α 3 were not recognized.

35 Factors that affected the binding affinity were identified as *i*) length of the *N*-acetyllactosamine carbohydrate chain, *ii*) branches of the carbohydrate chain, and *iii*) fucose substitution of the *N*-acetyllactosamine core chain.

5 1. N-acetyllactosamine core length: A preferential binding of *H. pylori* to NeuAc α 3-neolactooctaosylceramide over NeuAc α 3-neolactohexaosylceramide and NeuAc α 3-neolactotetraosylceramide was observed. This effect is most likely due to an improved accessibility of the carbohydrate head group when presented on a longer core chain.

10 2. Divalency: A cooperative binding may account for the increased affinity for NeuAc-G-10 ganglioside, having two NeuAc α 3Gal β 4GlcNAc β branches, relative to the linear NeuAc α 3-neolactohexaosylceramide. This is in agreement with the report 15 of Simon *et al.* (1997) demonstrating that multivalent albumin conjugates of sialyl-lactose (NeuAc α 3Gal β 4Glc) inhibited the adherence of *H. pylori* to epithelial monolayers more effectively than monovalent sialyl-lactose. The present invention shows that oligovalent or polyvalent presentation of sialyl-lactosamine allows effective representation this epitope

15 The lower binding affinity to the G9-B ganglioside relative to NeuAc α 3-neolactohexaosylceramide shows that the blood group B determinant on the β 6-linked branch interfered with the binding process. Still there is no absolute hindrance since the detection level for the G9-B ganglioside was approximately 100 pmole. 20 This suggests that the *H. pylori* binding determinants are mainly exposed on the β 3-axis of the Gal α 3(Fuc α 2)Gal β 4GlcNAc β 6(NeuAc α 3Gal β 4GlcNAc β 3)Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer structure.

25 3. Fucose branches on the N-acetyllactosamine core: The higher binding affinity for the VIM-2 ganglioside relative to NeuAc α 3-neolactohexaosylceramide, suggests that the α 3-linked Fuc at the innermost GlcNAc contributes to the high affinity binding of sialyl-dimeric- Le x . This fucose residue may either interact with the carbohydrate binding site of the SabA adhesin, or affect the conformation of the 30 ganglioside providing an optimal presentation of the head group. Resolution of this issue must, however, await the expression and crystallization of the SabA adhesin.

35 The expression of the SabA adhesin is, unlike the Le b -binding BabA adhesin, subjected to phase-variation (Mahdavi *et al.*, 2002). Varying number of bacteria expressing the SabA adhesin within the bacterial cell population used in the binding assays may account for the difficulties in determining an absolute affinity of binding for a given ganglioside. However, in repeated binding assays the same relationships between the binding-active gangliosides were observed. A noteworthy observation

is, however, that under optimal circumstances the binding of *H. pylori* to NeuAc α 3-neolactohexaosylceramide is comparable to the binding of cholera toxin B-subunits to the GM1 ganglioside. The choleratoxin binding is an example of very strong protein carbohydrate interaction.

5

The biological significance of these findings requires further studies. The sialic acid content of the primary target tissue of *H. pylori*, *i.e.* the human gastric epithelium, is very low (Madrid et al., 1990). However, it was recently demonstrated that an upregulation of the expression of sialic acid-containing glycoconjugates occurs upon 10 gastric inflammation (Mahdavi et al., 2002). Moreover, several of the *H. pylori* binding gangliosides are also present in human neutrophils (Miller-Podraza et al., 1999, Stroud et al., 1996 a, 1996b), and it was recently demonstrated that the nonopsonic *H. pylori*-induced activation of human neutrophils occurs by 15 lectinophagocytosis, *i.e.* recognition of sialylated glycoconjugates on the neutrophil cell surface by a bacterial adhesin leads to phagocytosis and an oxidative burst with the production of reactive oxygen metabolites (Teneberg, et al., 2001). Thus, the sialic acid binding capacity of *H. pylori* may have a dual role. On the one hand it mediates adhesion of bacteria to the epithelium in the already diseased stomach, and on the other leads to the activation of neutrophils to an oxidative burst with the 20 production of reactive oxygen metabolites and release of biologically active enzymes, giving rise to further tissue damage.

1. Binding of *H. pylori* to terminal NeuNAc α 3LacNAc on carbohydrate chains

We chose S-3PG as a model compound for our studies because this glycolipid is

25 relatively easy to prepare and represents a well defined structure. Recognition of sialic acid-containing glycoconjugates by some strains of *H. pylori* *in vitro* has been discussed in many papers (Karlsson 1998, Karlsson 2000, Miller-Podraza et al. 1997a, Johansson and Miller-Podraza 1998, Miller-Podraza et al 1996, Miller-Podraza et al. 1997b, Evans et al. 1988, Hirmo et al. 1996, Simon et al. 1997) 30 and our results are in line with these reports, strongly indicating the preference of *H. pylori* for α 3-linked NeuAc and β 4-linked Gal. There was apparently a requirement of neolacto structure, since NeuAc α 3Gal β 3GlcNAc-R and NeuAc α 3Gal β 3GalNAc-R were inactive. However, GlcNAc itself does not seem to be an absolute 35 requirement of the binding, because sialyllactose, NeuAc α 3Gal β 4Glc, was shown by others to weakly inhibit the interaction of *H. pylori* with sialylated structures (Evans et al. 1988, Hirmo et al. 1996, Simon et al. 1997). Besides, α 3 Fuc linked to GlcNAc (tested in sialyl-Lewis x oligosaccharide and glycolipid) and other modifications of GlcNAc like de-*N*-acetylation (Johansson and Miller-Podraza,

under preparation) do not abolish the binding. Thus, the present invention shows that modification of position 2 of Glc or GlcNAc is also possible for longer *H. pylori* binding oligosaccharides without loss of activity.

- 5 Derivatization of S-3-PG followed by binding studies showed dependence of the binding of *H. pylori* to sialylated epitopes on the glycerol tail and the carboxyl group. The importance of carboxyl group was demonstrated by reduction of COOH and synthesis of amides. Free amide and benzylamide showed some binding activity for *H. pylori* on TLC plates, however these reactions were not always reproducible
- 10 indicating reduced affinity. Large hydrophobic moiety in benzylamide and the positively charged NH₂ group in free amide could provoke unspecific bindings. On the other hand the binding to octadecylamide of SPG was at a lower pmol level (Fig. 4) indicating a specific interaction. The fact that free carboxyl may be replaced by amide form in octadecylamide indicates that only one oxygen of the carboxyl is
- 15 necessary for the interaction.

We think that NeuAcα3Galβ4GlcNAc, which is part of many human and animal glycoconjugates, represents the optimal terminal part of the natural sialylated binding saccharide for *H. pylori*. Of importance is that human neutrophils which are actively involved in *H. pylori* associated infections (Rautelin et al. 1993, Fiocca et al. 1994), are especially enriched in S-3-PG and other neolacto gangliosides (Fukuda et al. 1985, Müthing 1996, Stroud et al. 1996). As mentioned, S-3-PG was the simplest ganglioside from human neutrophils which bound *H. pylori* on our TLC plates. Fig. 11 shows, that there is a relatively stronger binding to more complex gangliosides. This strong interaction depends most probably on better presentation of the epitopes on TLC surface. During our work we have noticed that the length of the sugar chain as well as the structure of the lipid part may influence the binding. For example, binding of *H. pylori* to neoglycolipids with hexadecylaniline was less reproducible than binding to neoglycolipids with branched lipid chains and the binding to sialoneohexaosylceramide was stronger than binding to sialoneotetraosylceramide, as judged from TLC plates (Fig. 11). However other factors should also be taken into account like repeated epitope units, fucose branches or other substitutions. The strong binding to larger species may also depend on combined binding of different bacterial adhesins recognizing both terminal and internal parts the extended core chains. *H. pylori* is known to display several different binding specificities associated with both sialylated and neutral saccharide chains (Karlsson 1998). Recently Roche N. et al. reported binding of *H. pylori* to gangliosides with repeated lactosamine units prepared from human gastric carcinoma (Roche et al. 2001).

2. Binding epitope associated with branched poly-*N*-acetyllactosamine chains

We have previously shown that NeuAc in *H. pylori*-binding PGCs is associated with short branches based on one lactosamine unit (NeuAc-Hex-HexNAc). This was

5 proven by digestion of PGCs by endo- β -galactosidase and analysis of the released oligosaccharide fragments using various mass spectrometry techniques (Karlsson et al. 2000). The presence of NeuAc α 3Gal β 4GlcNAc in branched PGCs provides favourable conditions for the formation of hydrogen bonds between different sugars and a hypothesis was established that a new binding epitope is created based on
10 interaction between C9 of the glycerol tail of NeuAc and GlcNAcs of the two neighbouring branches (Ångström et al, submitted). Such hypothesis is in agreement with the importance of the glycerol tail for the interaction and can explain the apparent existence of two different binding modes of *H. pylori* to sialylated structures. The present paper confirms our previous findings on the importance of
15 the glycerol group (Miller-Podraza et al.1996) and shows that even carboxyl group is crucial for the binding. This is interesting since COOH provides possibilities of rapid *in vivo* switching off and on of the binding by lactonization or other reversible modifications.

TABLE I. Ganglioside library and results on *Helicobacter pylori* binding activity

No.	Trivial name	Structure	CCUG 17874.	Source
1.	NeuAc-GM3	NeuAc α 3Gal β 4Glc β 1Cer	- ^a	Human meconium
2.	NeuAc α 3SPG	NeuAc α 3Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer	+	Human erythrocytes
3.	NeuAc α 6SPG	NeuAc α 6Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer	-	Human meconium
4.	NeuG α 3SPG	NeuG α 3Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer	-	Rabbit thymus
5.	NeuAc-DPG	NeuAc α 8NeuAc α 3Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer	-	Human kidney
6.	NeuAc α 3-L α^a	NeuAc α 3Gal β 3(Fuc α 4)GlcNAc β 3Gal β 4Glc β 1Cer	-	Human gallbladder cancer
7.	NeuAc α 3-L α^x	NeuAc α 3Gal β 4(Fuc α 3)GlcNAc β 3Gal β 4Glc β 1Cer	+	Commercial
8.	NeuAc α 3-nLc6	NeuAc α 3Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer	+++	Human hepatoma
9.	NeuG α 3-nLc6	NeuG α 3Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer	-	Rabbit thymus
10.	NeuAc α 3-nLc8	NeuAc α 3Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer	+++	Human erythrocytes
11.	NeuG α 3-nLc8	NeuG α 3Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer	-	Rabbit thymus
12.	VIM-2	NeuAc α 3Gal β 4GlcNAc β 3Gal β 4(Fuc α 3)GlcNAc β 3Gal β 4Glc β 1Cer	+++	Human colon cancer
13.	S-dimer-L α^x	NeuAc α 3Gal β 4(Fuc α 3)GlcNAc β 3Gal β 4(Fuc α 3)GlcNAc β 3Gal β 4Glc β 1Cer	-	Human gallbladder cancer
14.		Gal β 4GlcNAc β 6(NeuAc α 6Gal β 4GlcNAc β 3)Gal β 4Glc β 1Cer	-	Bovine buttermilk
15.		Gal β 4GlcNAc β 6(NeuAc α 6Gal β 4GlcNAc β 3)Gal β 4Glc β 1Cer	-	Human meconium
16.	NeuAc-C-10	NeuAc α 3Gal β 4GlcNAc β 6(NeuAc α 3Gal β 4GlcNAc β 3)Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer	+++	Human erythrocytes

Table 1 (Continued)

17. NeuGc-G-10 NeuGc α 3Gal β 4GlcNAc β 6(NeuGc α 3Gal β 4GlcNAc β 3)Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer Bovine erythrocytes

18. Gal α 3Gal β 4GlcNAc β 6(NeuGc α 3Gal β 4GlcNAc β 3)Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer Bovine erythrocytes

19. G9-B Gal α 3(Fuc α 2)Gal β 4GlcNAc β 6(NeuAc α 3Gal β 4GlcNAc β 3)Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer Human erythrocytes

a) Binding is defined as follows: +++ denotes a binding when less than 0.5 μ g of the glycosphingolipid was applied on the thin-layer chromatogram, while + denotes an occasional binding at 0.5 μ g, and - denotes no binding even at 4 μ g.

S-3-PG derivative	Chemical modification	M-H Found	Sialic acid fragment Found	Calculated (accurate)	Binding
Unmodified.		1629.0	291.2	291.1	+
Reduced	R-COOH -> R-CH ₂ OH	1615.3	277.4	277.1	-
Amide	R-COOH -> R-CONH ₂	1628.0	290.2	290.1	(+)
Methylamide	R-COOH -> R-CONH-CH ₃	1641.9	304.2	304.1	-
Ethylamide	R-COOH -> R-CONH-CH ₂ CH ₃	1656.3	318.5	318.1	-
Propylamide	R-COOH -> -> R-CONH-CH ₂ CH ₂ CH ₃	1670.2	332.3	332.2	-
Benzylamide	R-COOH -> R-CONH-CH ₂ C ₆ H ₅	1718.5	380.7	380.2	(+)
Octadecylamide	R-COOH -> R-CONH-(CH ₂) ₁₇ CH ₃	1880.1	542.3	542.6	+
Oxidized/reduced	R-CHOH-CHOH-CH ₂ OH -> -> R-CHOH-CH ₂ OH + + R-CH ₂ OH	1599.2 1569.8	261.3 231.9	261.1 231.1	-
Oxidized/coupled with CH ₃ NH ₂	R-CHOH-CHOH-CH ₂ OH -> -> R-CH ₂ NH-CH ₃	1582.3	244.1	244.1	-
Oxidized/coupled with OHCH ₂ CH ₂ NH ₂	R-CHOH-CHOH-CH ₂ OH -> -> R-CH ₂ NH-CH ₂ CH ₂ OH	1612.2	274.3	274.1	-
As lactone		1611.2	273.3	273.1	-

TABLE 3. Binding of *Helicobacter pylori* to various glycolipids on TLC plates. HDA, hexadecylaniline

Glycolipid	Binding of <i>H. pylori</i>	Source
NeuAc α 3Gal β 4GlcNAc β 3Gal β 4GlcCer, S-3-PG	+	Human erythrocytes
NeuAc α 3Gal β 4(Fuc α 3)GlcNAc β 3Gal β 4GlcCer (Sialyl-Lewis ^x)	+	Synthesis
NeuAc α 6Gal β 4GlcNAc β 3Gal β 4GlcCer, S-6-PG	-	Human leukocytes
NeuAc α 3Gal β 3GlcNAc β 3Gal β 4Glc-HDA (or -branched lipid)	-	Chemical synthesis
NeuAc α 6Gal β 4GlcNAc β 3Gal β 4Glc-HDA (or -branched lipid)	-	Chemical synthesis
Gal β 3(NeuAc α 6)GlcNAc β 3Gal β 4Glc-HDA (or -branched lipid)	-	Chemical synthesis
NeuAc α 3Gal β 4GlcNAc β 3Gal β 4Glc-HDA (or -branched lipid)	-	Chemical synthesis
NeuAc α 3Gal β 4GlcNAc β 3Gal β 4Glc-HDA (or -branched lipid)	+	Human erythrocytes
NeuAc α 3Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4GlcCer	+	Bovine brain
NeuAc α 3Gal β 3GlcNAc β 4(NeuAco β 3)Gal β 4GlcCer, GD1a	-	Human erythrocytes
NeuAc α 8NeuAco β 3Gal β 4GlcNAc β 3Gal β 4GlcCer	-	Bovine brain
Gal β 3GalNAc β 4(NeuAco β 3)Gal β 4GlcCer, GM1a	-	Enzymatic synthesis
NeuAc α 3Gal β 3GlcNAc β 4Gal β 4GlcCer, GM1b	-	Bovine brain
Gal β 3GalNAc β 4(NeuAco β 8NeuAco β 3)Gal β 4GlcCer, GD1b	-	Bovine brain
NeuAc α 3Gal β 3GlcNAc β 4(NeuAco β 8NeuAco β 3)Gal β 4GlcCer	-	Bovine brain
NeuAc α 8NeuAco β 3Gal β 3GlcNAc β 4(NeuAco β 8NeuAco β 3)Gal β 4GlcCer, GQ1b	-	Human brain

Table 4. Binding of *H. pylori* (032 strain cultivated in broth) to derivatized PGCs. R stands for the rest of the PGC molecule.

PGC Preparation	Chemical modification	Binding
Unmodified		
Reduced	R-COOH → R-CH ₂ OH	+
Oxidized/reduced	R-CHOH-CHOH-CH ₂ OH → R-CHOH-CH ₂ OH + R-CH ₂ OH	-
Oxidized/coupled with OHCH ₂ CH ₂ NH ₂	R-CHOH-CHOH-CH ₂ OH → R-CH ₂ -NH-CH ₂ -CH ₂ OH	-

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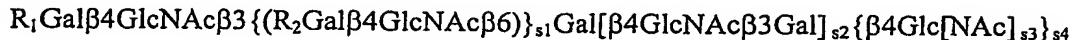
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20

What is claimed:

1. A *Helicobacter pylori* binding substance comprising an oligosaccharide sequence according to Formula 1

5

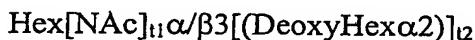


wherein R1 and R2 are terminal mono-or oligosaccharides substituents so that at least one of the substituents is NeuNAc α 3; s1, s2, s3 and s4 are independently

10 integers 0 or 1 indicating presence or absence of the structure in {} or in [];

as a non-reducing end terminal sequence, and *Helicobacter pylori* binding analogs and derivatives thereof, for use as a medicament.

15 2. The substance according to claim 1, wherein R1 or R2, when not being NeuNAc α 3, indicates terminal substituents linked to position 2 and/or 3 of the terminal Gal according to Formula 2



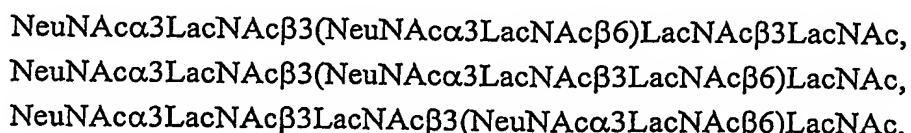
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wherein Hex is preferably Gal or Glc, integers t1 and t2 are independently 0 or 1 and α/β means that the linkage is either α or β .

25 3. The substance according to claim 2, wherein non-sialylated R1 or R2 is a structure selected from the group consisting of Gal α 3, GalNAc α 3, Fuca2, Gal α 3(Fuca2), GalNAc α 3(Fuca2), Gal β 4GlcNAc β 3, GlcNAc β 3Gal β 4GlcNAc β 3, Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3, GlcNAc α 3, GlcNAc β 3, GalNAc β 3, Gal β 3, Glc β 3, and Glc α 3.

30 4. The substance according to claim 2, wherein non-sialylated R1 or R2 is a structure selected from the group consisting of blood group antigen type structures: Gal α 3, GalNAc α 3, Fuca2, Gal α 3(Fuca2), and GalNAc α 3(Fuca2).

35 5. The substance according to claim 1, wherein said substance is



5 NeuNAc α 3LacNAc β 3(NeuNAc α 3LacNAc β 6)LacNAc β 3Lac,
 NeuNAc α 3LacNAc β 3(NeuNAc α 3LacNAc β 6)LacNAc β 3Gal,
 NeuNAc α 3LacNAc β 3(NeuNAc α 3LacNAc β 6)Lac,
 NeuNAc α 3LacNAc β 3(NeuNAc α 3LacNAc β 6)LacNAc, or
 NeuNAc α 3LacNAc β 3(NeuNAc α 3LacNAc β 6)Gal

6. The substance according to claim 1, wherein the oligosaccharide sequences comprise further poly-N-acetyllactosamine branches.

10 7. The substance according to claim 6 having the structure

NeuNAc α 3LacNAc β 3(NeuNAc α 3LacNAc β 6)LacNAc β 3(NeuNAc α 3LacNAc β 6)LacNAc

15 wherein LacNAc indicates N-acetyllactosamine, Gal β 4GlcNAc, and Lac is lactose, Gal β 4Glc.

20 8. The substance according to claim 1, wherein s1 is 0 and said substance is

25 NeuNAc α 3LacNAc β 3LacNAc β 3LacNAc,
 NeuNAc α 3LacNAc β 3LacNAc β 3Lac,
 NeuNAc α 3LacNAc β 3LacNAc β 3Gal,
 NeuNAc α 3LacNAc β 3LacNAc,
 NeuNAc α 3LacNAc β 3Lac, or
 NeuNAc α 3LacNAc β 3Gal

30 9. The substance according to claim 1, wherein at least one of N-acetyllactosamine residues have been replaced by type 2 N-acetyllactosamine analogous structure or structures, preferably by lactose residues according to Formula 3

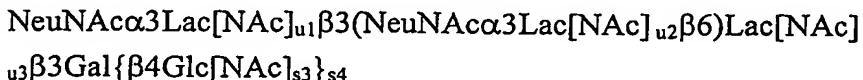
$R_1Gal\beta 4Glc[NAc]_{u1}\beta 3\{(R_2Gal\beta 4Glc[NAc]_{u2}\beta 6)\}_{s1}Gal\{\beta 4Glc[NAc]_{u3}\beta 3Gal\}$
 $s_2\{\beta 4Glc[NAc]_{s3}\}_{s4}$

35 wherein R1 and R2 are independently nothing or terminal mono- or oligosaccharides substituents with the proviso that at least one of the substituents is NeuNAc α 3 or NeuNAc α 3Gal β 4Glc[NAc] $_{u4}\beta 3$; integers s1, s2, s3 and s4 are independently 0 or 1,

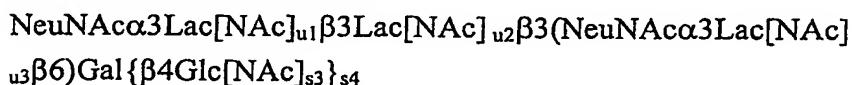
indicating the presence or absence of the structures in [] or in {}; integers u1, u2, u3, and u4 are independently 0 or 1 indicating the presence or absence of the N-acetyl groups in the non-reducing end terminal or midchain lactosamine residues with the proviso that at least one of the integers present is 0.

5

10. The substance according to claim 9, wherein said substance is:



10 NeuNAc α 3Lac[NAc] $_{u1}$ β 3(NeuNAc α 3Lac[NAc] $_{u2}$ β 3Lac[NAc] $_{u3}$ β 6)Gal $\{\beta$ 4Glc[NAc] $_{s3}\}_{s4}$



15 NeuNAc α 3Lac[NAc] $_{u1}$ β 3Lac[NAc] $_{u2}$

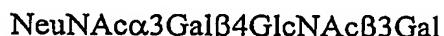
11. The substance according to claim 1, wherein said substance is further fucosylated according to the formula 4

20 R₁Gal β 4[(Fuc α 3)]_{t1}Glc[NAc] $_{u1}$ β 3{(R₂Gal β 4[(Fuc α 3)]_{t2}Glc[NAc] $_{u2}$ β 6)}_{s1}Gal[β 4[(Fuc α 3)]_{t3}Glc[NAc] $_{u3}$ β 3Gal]_{s2}{ β 4[(Fuc α 3)]_{t4}Glc[NAc] $_{s3}\}_{s4}$

wherein R₁ and R₂ are independently nothing or terminal mono- or oligosaccharides substituents with the proviso that at least one of the substituents is NeuNAc α 3 or

25 NeuNAc α 3Gal β 4[(Fuc α 3)]_{t5}Glc[NAc] $_{u4}\beta$ 3; integers s₁, s₂, s₃, and s₄ are independently 0 or 1 indicating the presence or absence of the structures in [] or in {}; integers u₁, u₂, u₃, and u₄ are independently 0 or 1 indicating the presence or absence of the N-acetyl groups in the non-reducing end terminal or midchain lactosamine residues with the proviso that at least one of the integers present is 0; integers t₁, t₂, t₃, t₄ and t₅ are independently 0 or 1 indicating the presence or absence of the Fuc α 3-branch-structures in [] so that at least t₁, t₂, t₃, t₄ or t₅ is 1.

30 35 12. The substance according to claim 1, wherein said substance comprises an oligosaccharide sequence according to Formula 5



13. The substance according to claim 1, wherein said substance comprises an oligosaccharide sequence according to Formula 6



5

wherein m is 0 or 1.

14. The substance according to claim 12 or 13, wherein the oligosaccharide sequence is not pentasaccharide glycolipid NeuNAc α 3Gal β 4GlcNAc β 3Gal β 4Glc β Cer.

10

15. The substance according to claim 12 or 13, wherein the oligosaccharide sequence is not linked to ceramide or a hydrophobic aglycon or spacer comprising more than 22 carbon atoms.

15

16. The substance according to claim 12 or 13, wherein the tetrasaccharide sequence is coupled to an aglycon or spacer comprising less than 8 carbon atoms in a hydrophobic structure.

20

17. The substance according to any one of claims 1-16, wherein said substance is conjugated to a polysaccharide, preferably to a polylactosamine chain or a conjugate thereof.

18. The substance according to any one of claims 1-16, wherein said substance is a glycolipid.

25

19. The substance according to any one of claims 1-16, wherein said substance is an oligomeric molecule containing at least two or three oligosaccharide chains.

30

20. The substance according to any one of claims 1-16, wherein said substance consists of a micelle comprising one or more of the substances as defined in claims 1 - 16.

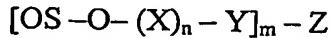
21. The substance according to any one of claims 1 – 20, wherein said substance(s) is/are conjugated to a carrier.

35

22. The substance according to any one of claims 1 - 16, wherein said substance is covalently conjugated with an antibiotic effective against *Helicobacter pylori*, preferably a penicillin type antibiotic.

23. The substance according to claim 21, wherein position C1 of reducing end terminal Gal, Glc or GlcNAc of said oligosaccharide sequence (OS) is oxygen linked (-O-) to an oligovalent or a polyvalent carrier (Z), via a spacer group (Y) and optionally via a monosaccharide or oligosaccharide residue or derivative (X),

5 forming the following structure



where integers m, and n have values $m \geq 1$, and n is independently 0 or 1; X is preferably lactosyl-, galactosyl-, poly-N-acetyl-lactosaminyl, or part of an O-glycan or an N-glycan oligosaccharide sequence, Y is a spacer group or a terminal conjugate such as a ceramide lipid moiety or a linkage to Z;

or a derivative of the substance of said structure having binding activity to
15 *Helicobacter pylori*.

24. A pharmaceutical composition comprising a substance of any one of claims 1-22 for the treatment or prophylaxis of any condition due to the presence of *Helicobacter pylori*.

20

25. A pharmaceutical composition according claim 24, wherein said pharmaceutical composition is for the treatment of chronic superficial gastritis, gastric ulcer, duodenal ulcer, gastric adenocarcinoma, non-Hodgkin lymphoma in human stomach, liver disease, pancreatic disease, skin disease, heart disease, or autoimmune diseases including autoimmune gastritis and pernicious anaemia and non-steroid anti-inflammatory drug (NSAID) related gastric disease, or for prevention of sudden infant death syndrome.

26. Use of the substance as defined in claims 1 - 23 for the production of a
30 pharmaceutical or nutritional composition for the treatment or prophylaxis of any condition due to the presence of *Helicobacter pylori*.

27. Use of the substance as defined in claims 1 - 23, for the diagnosis of a condition due to infection by *Helicobacter pylori*.

35

28. A nutritional additive, food-stuff or beverage containing the composition or substance according to any one of claims 1 – 23.

29. The nutritional additive according to claim 28 for use in infant food.

30. A method for the treatment of a condition due to presence of *Helicobacter pylori*, wherein a pharmaceutically effective amount of the substance as defined in any one of claims 1 – 23 is administered to a subject in need of such treatment.

5 31. The method according to claim 30, when said condition is caused by the presence of *Helicobacter pylori* in the gastrointestinal tract of a patient.

32. The method according to claim 30 for the treatment of chronic superficial gastritis, gastric ulcer, duodenal ulcer, gastric adenocarcinoma, non-Hodgkin lymphoma in human stomach, liver disease, pancreatic disease, skin disease, heart disease, or autoimmune diseases including autoimmune gastritis and pernicious anaemia and non-steroid anti-inflammatory drug (NSAID) related gastric disease, or for prevention of sudden infant death syndrome.

10 33. The method of treatment according to any one of claims 30 – 32, wherein said substance is a nutritional additive or a part of a nutritional composition.

15 34. The composition or substance according to any one of claims 1 – 23 for binding or inhibition of *Helicobacter pylori*.

20 35. Use of the substance as defined in claims 1 - 23 for the production of a nutritional additive or composition for the treatment or prophylaxis of any condition due to the presence of *Helicobacter pylori*.

25 36. The use according to claim 35 wherein said nutritional additive or composition is for infant food.

30 37. Use of the substance as defined in claims 1 – 23, for the identification of bacterial adhesin.

35 38. Use of the substance as defined in claims 1 – 23 or a substance identified according to claim 34, for the production of a vaccine against *Helicobacter pylori*.

39. Use of the substance as defined in claims 1 – 23 for typing *Helicobacter pylori*.

40. Use of the substance as defined in claims 1 – 23 for *Helicobacter pylori* binding assays.

41. The *Helicobacter pylori* binding non-acidic polyvalent substance according to claim 23, wherein linker structure Y is

5 [OS -O- (X)_n-L₁-CH(H/{CH₁₋₂OH}_{p1}) - {CH₁OH} _{p2}- {CH(NH-R)} _{p3} - {CH₁OH} _{p4} - L₂]_m-Z

wherein L₁ and L₂ are linking groups comprising independently oxygen, nitrogen, sulphur or carbon linkage atom or two linking atoms of the group forming linkages such as -O-, -S-, -CH₂-, -N-, -N(COCH₃)-, amide groups -CO-NH- or -NH-CO- or 10 -N-N- (hydrazine derivative) or an amino oxy-linkages -O-N- and -N-O-; L₁ is linkage from carbon 1 of the reducing end monosaccharide of X or when n =0, L₁ replaces -O- and links directly from the reducing end C1 of OS; p1, p2, p3, and p4 are independently integers from 0-7, with the proviso that at least one of p1, p2, p3, and p4 is at least 1; CH₁₋₂OH in the branching term {CH₁₋₂OH}_{p1} means that the 15 chain terminating group is CH₂OH and when the p1 is more than 1 there is secondary alcohol groups -CHOH- linking the terminating group to the rest of the spacer; R is preferably acetyl group (-COCH₃) or R is an alternative linkage to Z and then L₂ is one or two atom chain terminating group, in another embodiment R is 20 an analog forming group comprising C₁₋₄ acyl group comprising amido structure or H or C₁₋₄ alkyl forming an amine; and m > 1 and Z is polyvalent carrier; OS and X are as defined in claim 12.

42. A *Helicobacter pylori* binding substance comprising a sialic acid derivative as a non-reducing end terminal sequence with binding affinity towards *Helicobacter pylori* having the structure 25

SA(X-R)

wherein X is a linking atom or group bound to C1 of sialic acid, R is H or an organic 30 radical comprising more than 3 carbon atoms; X is preferably -NH forming amide structure with the carboxylic acid group of the sialic acid residue; R is preferably H or a C₄₋ C₃₀ organic radical comprising a ring structure and/or an aliphatic chain; R is more preferably a C₆-C₂₄ organic radical and most preferably R is a C₆₋₂₄ aliphatic alkyl chain

35

43. The substance according to claim 42, wherein said sialic acid is NeuNAc.

44. The substance according to claim 42, wherein said sialic acid is α 3-linked to type two N-acetyllactosamine sequence having the structure



wherein x is linkage position of the sialic acid derivative and

5 integers p1, p2 and p3 are independently 0 or 1 indicating the presence or absence of the whole structure in { }, [] or ().

45. The substance according to any one of claims 42-44 for use as a medicament.

10 46. The substance as defined in claims 9-11.

47. A soluble polyvalent substance comprising at least two oligosaccharide sequences from different groups defined in any of the claims 1-23 or 42-44.

15 48. A food preservative comprising at least one oligosaccharide sequences defined in any of the claims 1-23 or 42-44.

20 49. A mouth hygiene product comprising at least one oligosaccharide sequence defined in any of the claims 1-23 or 42-44.

50. A mouth hygiene product according to the claim 49 when the product is selected from group consisting of: tooth pastes, mouth wash solutions, tablets, and chewing gums.

25 51. A topical, washing or cosmetic product comprising at least one of the oligosaccharide sequences defined in any of the claims 1-23 or 42-44.

30 52. A topical, washing or cosmetic product according to the claim 51 when the product is selected from the group consisting of: tooth pastes, mouth wash solutions, tablets, cleanser, disinfectant and chewing gums.

53. Use of a composition defined in any of the claims 1-23 or 42-44 for non-diagnostic inhibition or agglutination of pathogen *ex vivo*.

35 54. Use according to claim 63 when the pathogen is *H. pylori*.

(57) Abstract

The present invention describes an oligosaccharide substance or receptor binding to *Helicobacter pylori*, and the use thereof in, e.g., pharmaceutical and nutritional compositions for the treatment of conditions due to the presence of *Helicobacter pylori*. The invention is also directed to the use of the receptor for diagnostics of *Helicobacter pylori*.

Fig. 1A

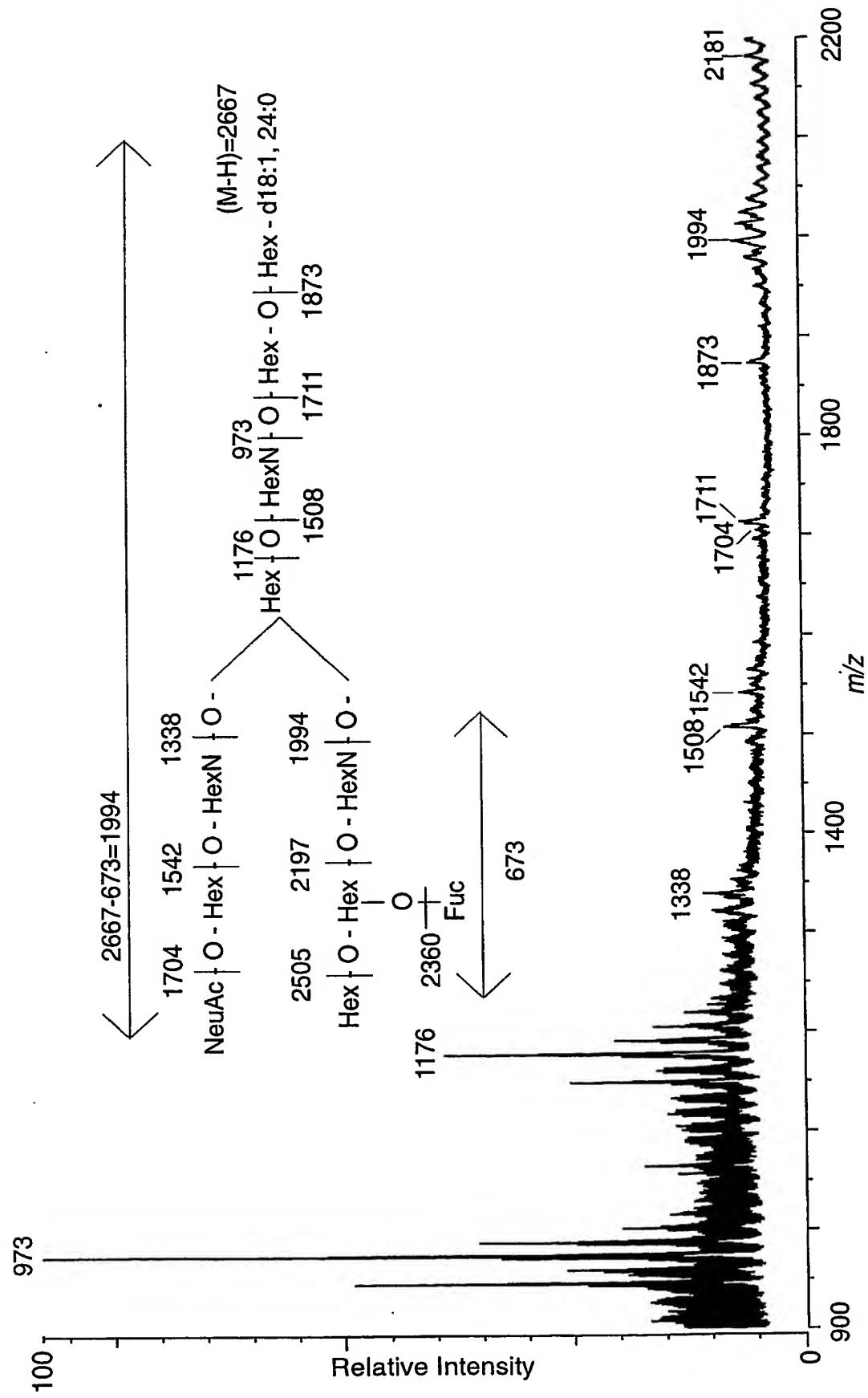
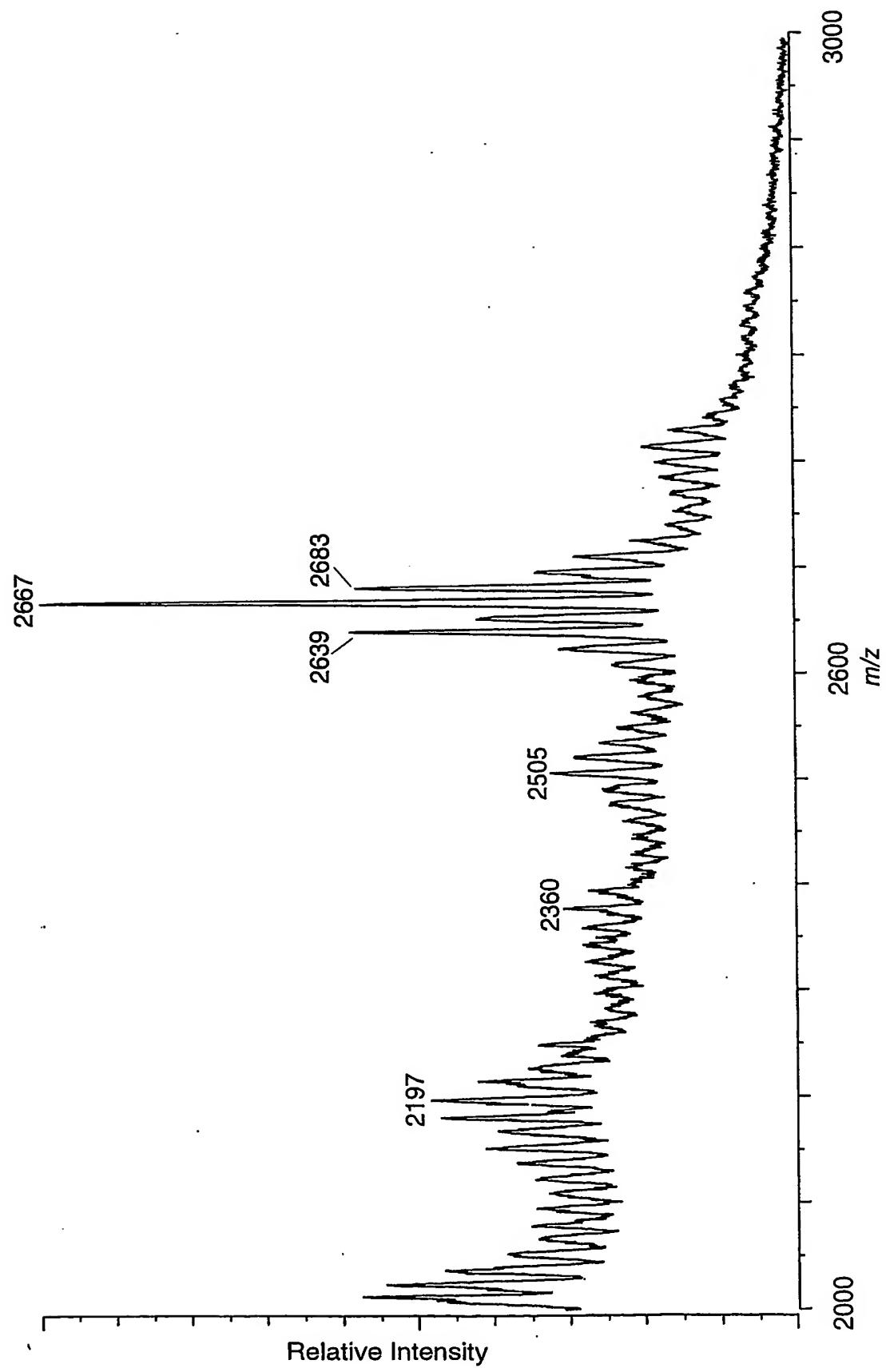


Fig. 1B



L5
3

Fig. 2

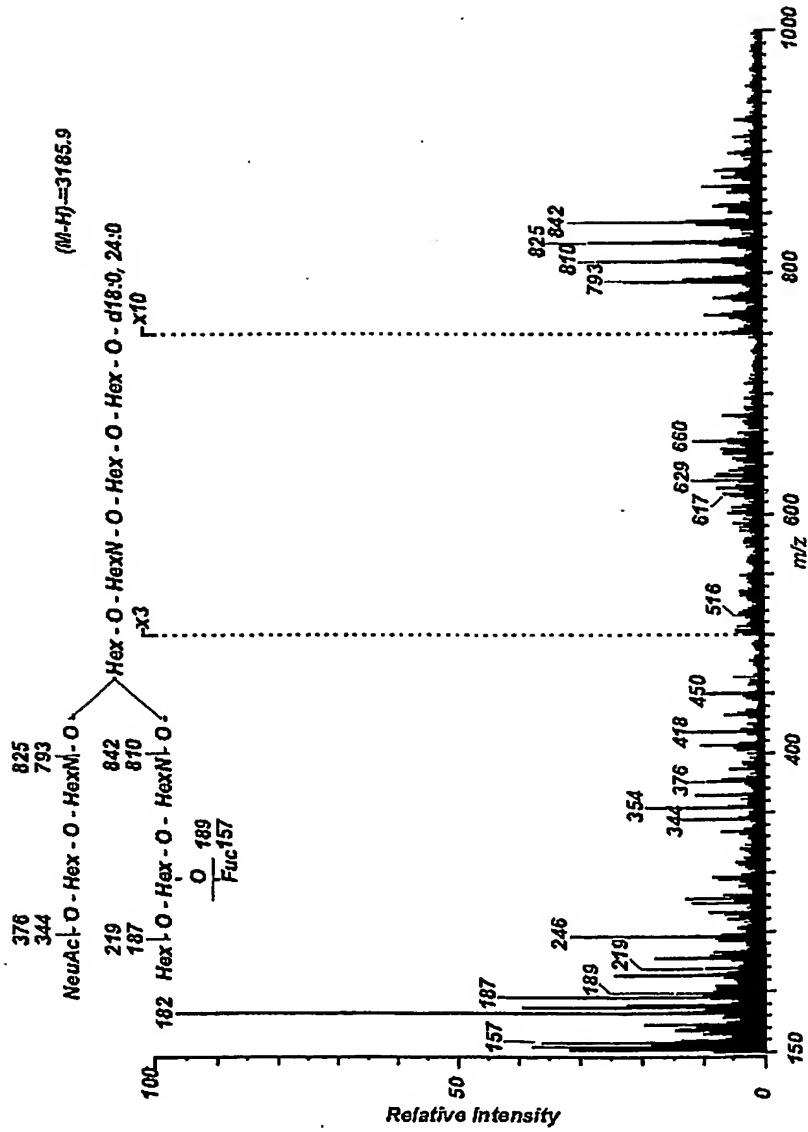
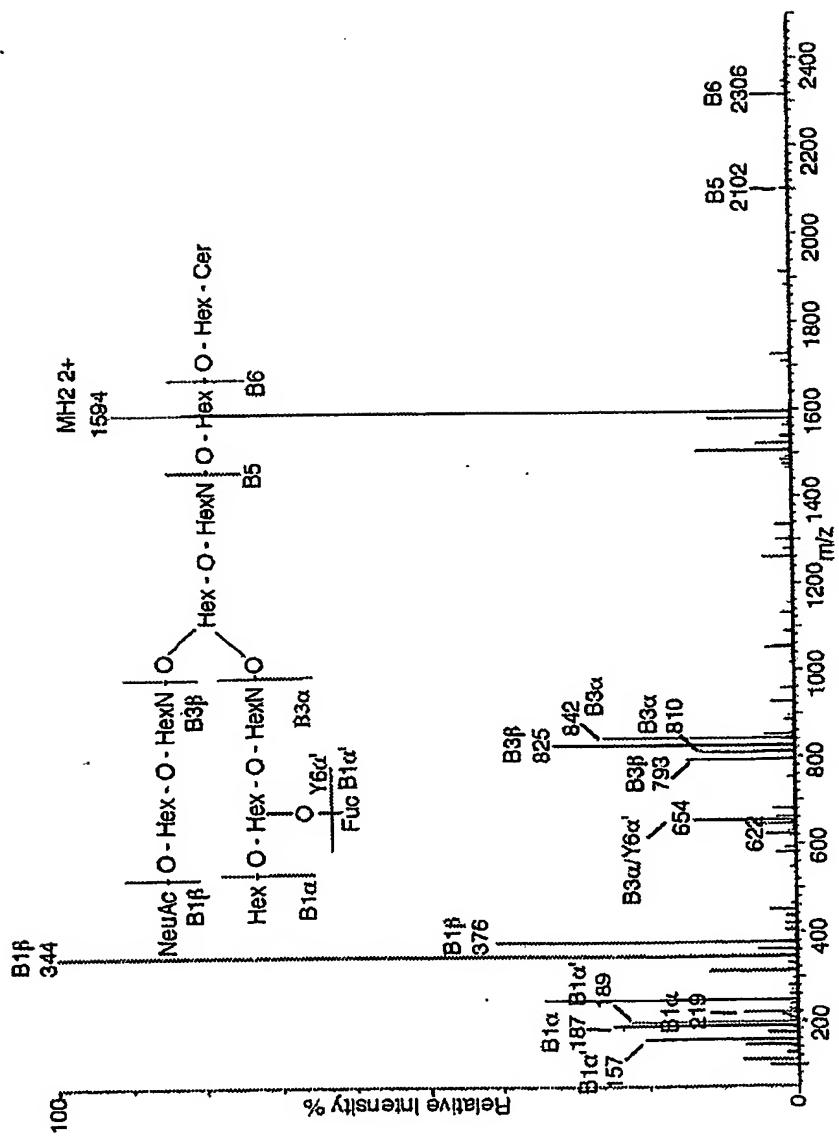


Fig. 3



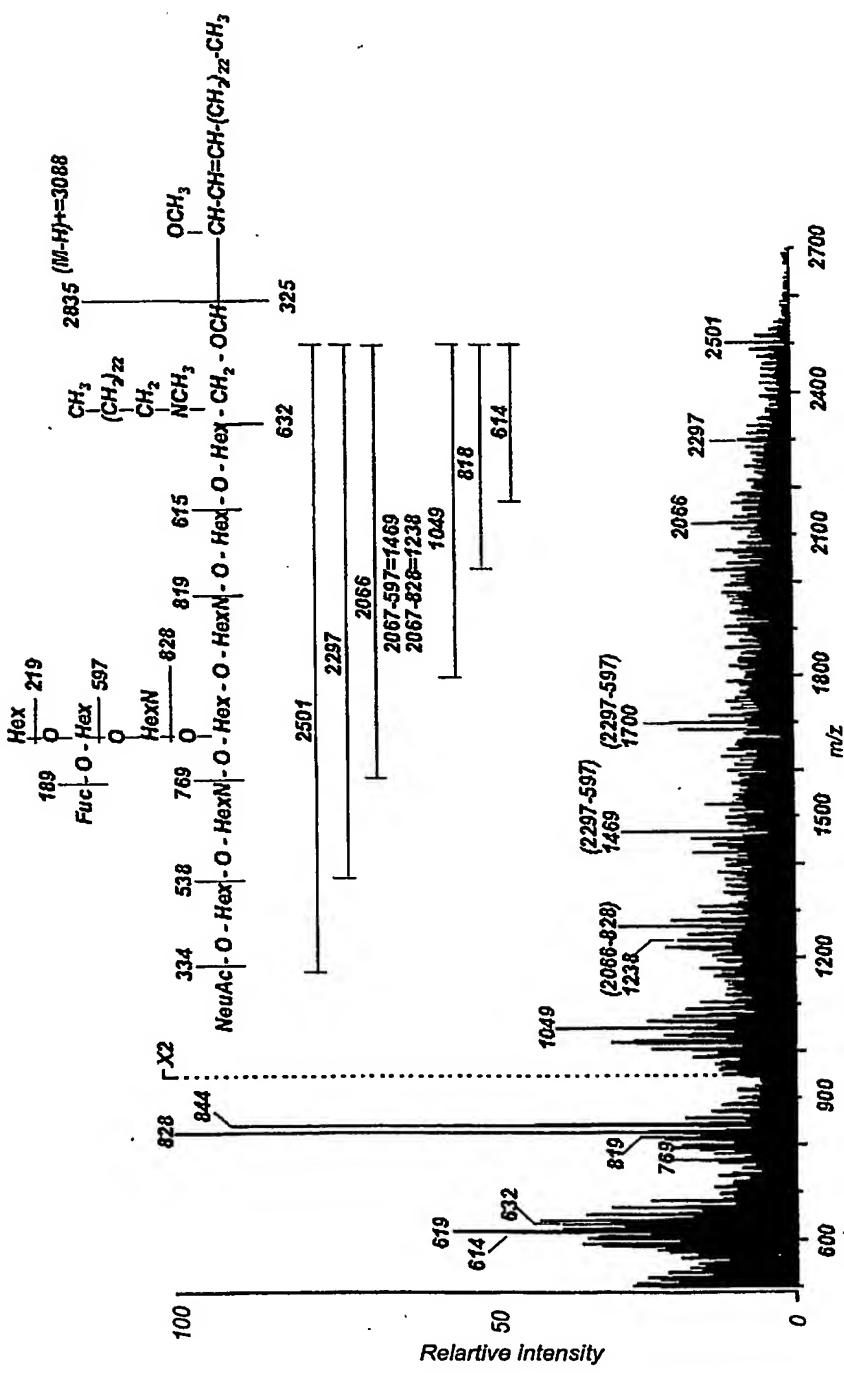


Fig. 4

Fig. 5
A.

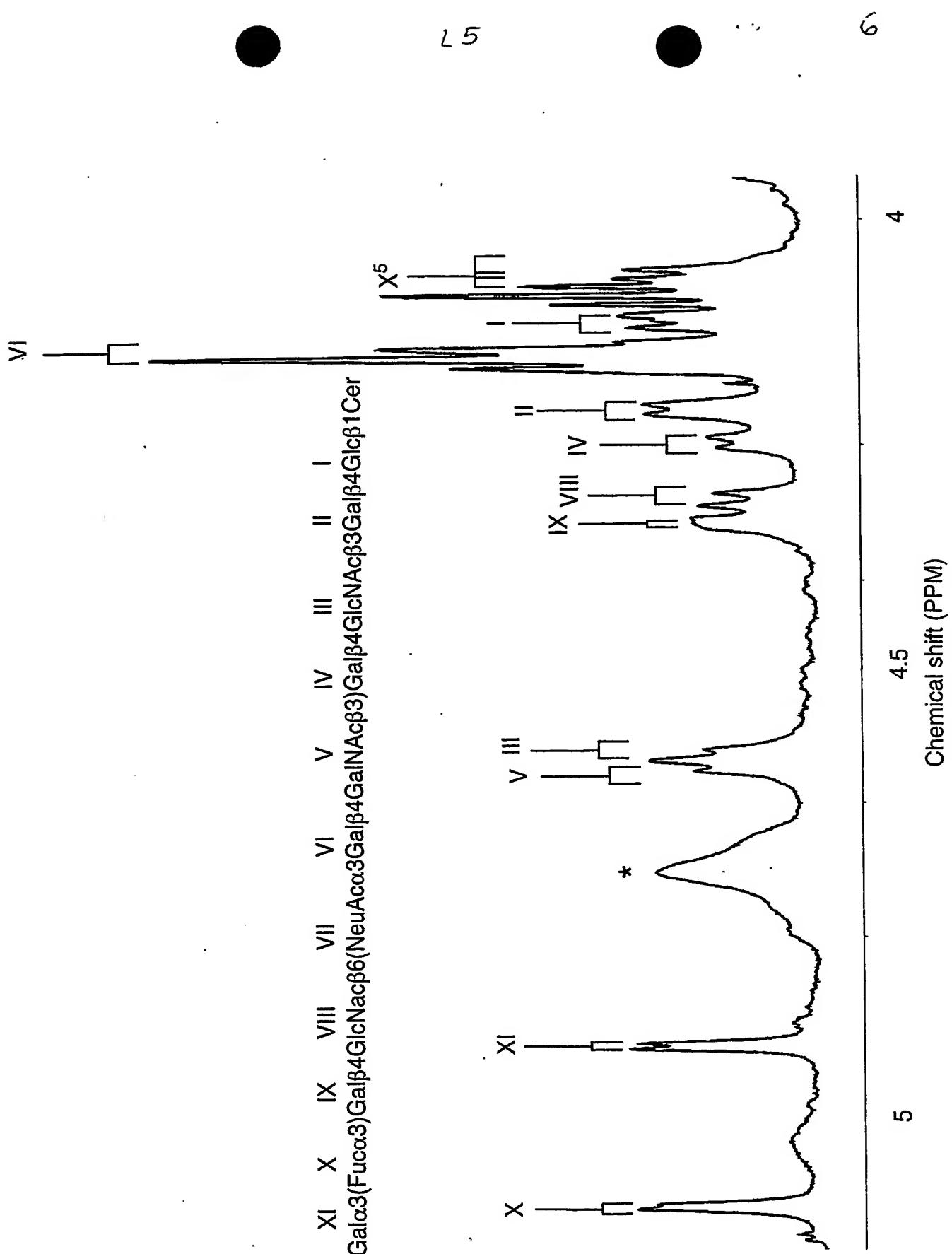


Fig. 5B

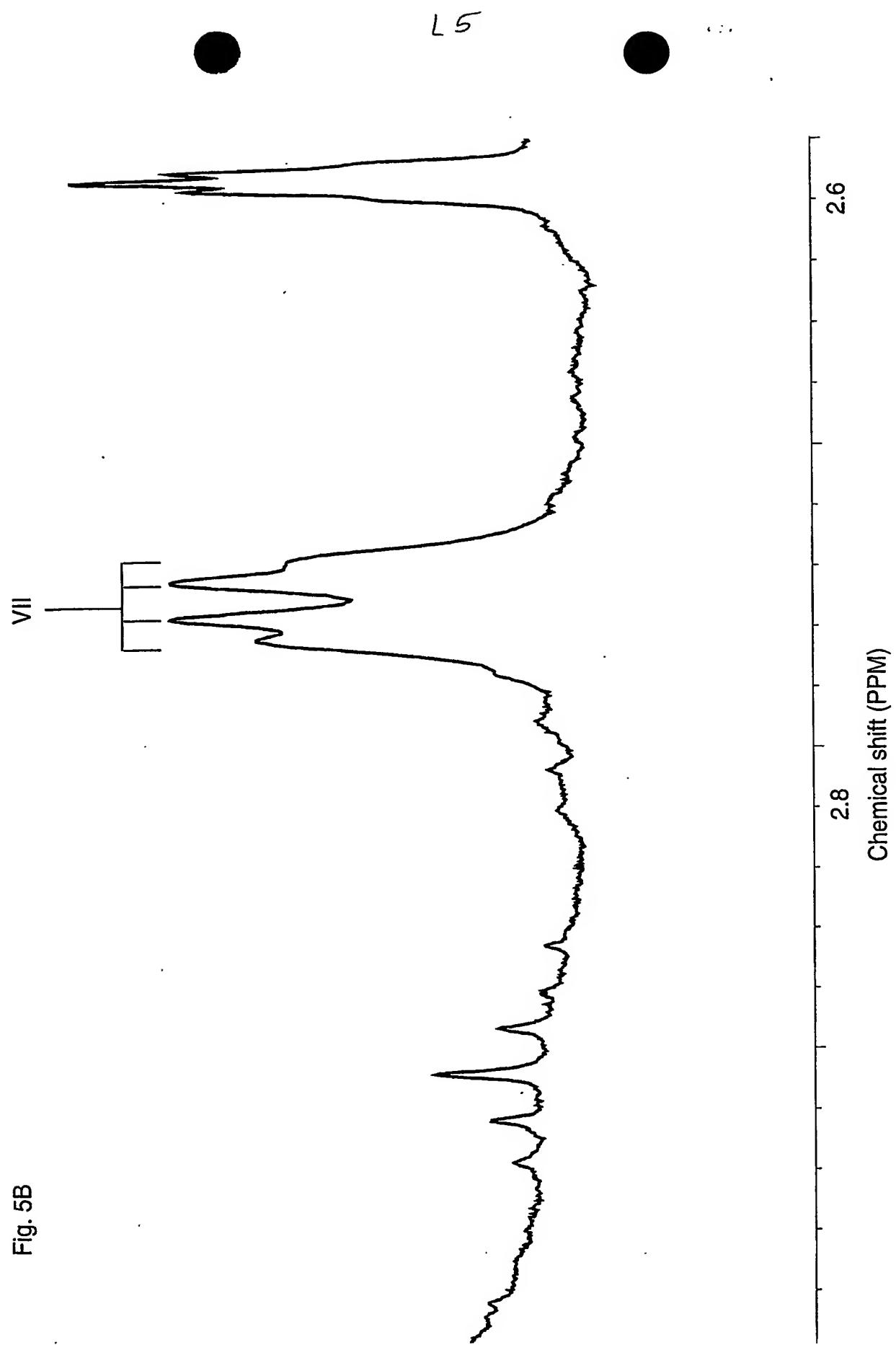


Fig. 6

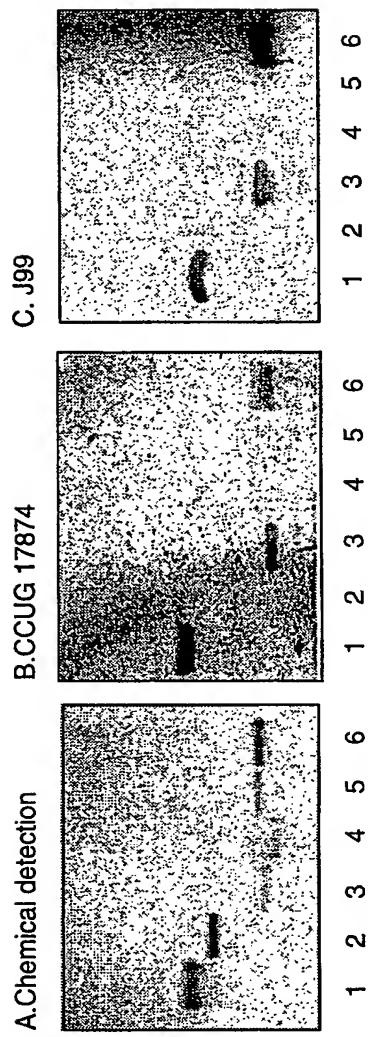
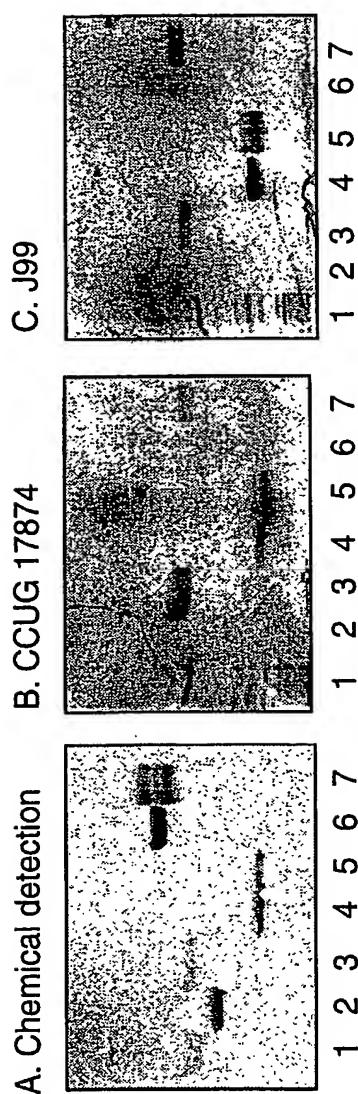


Fig. 7



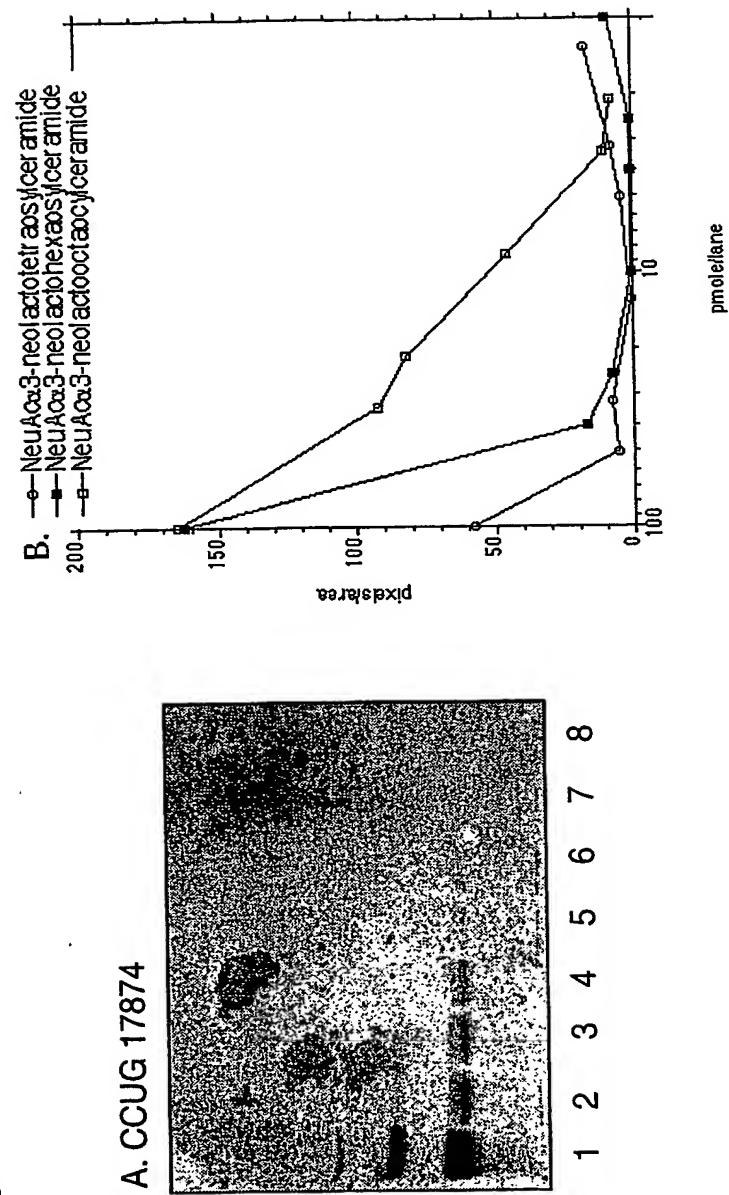
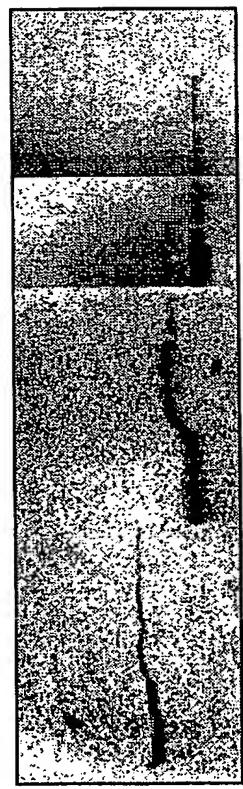


Fig. 8

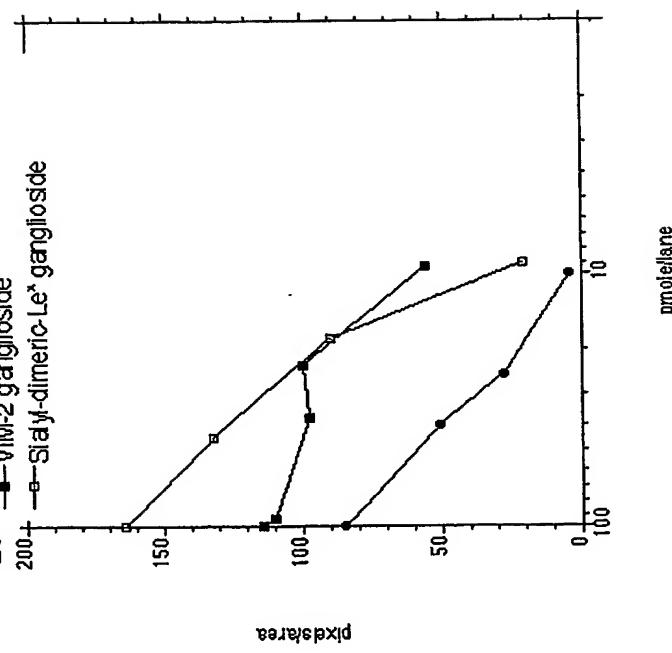
Fig. 9

A. CCUG 17874



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

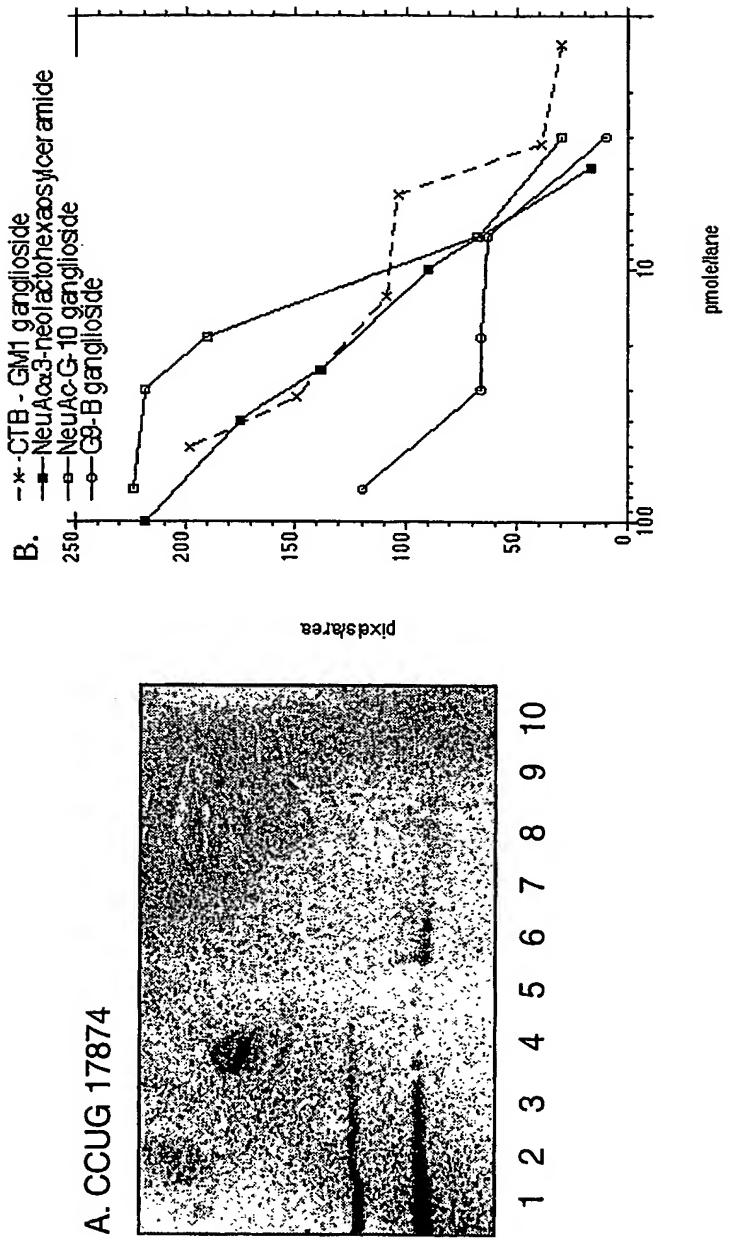
B.
—●— NeuA α 2-neolactohexaosylceramide
—■— VIM-2 ganglioside
—□— Sialyldimeric-Lewis α ganglioside



L5

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Fig. 10



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12

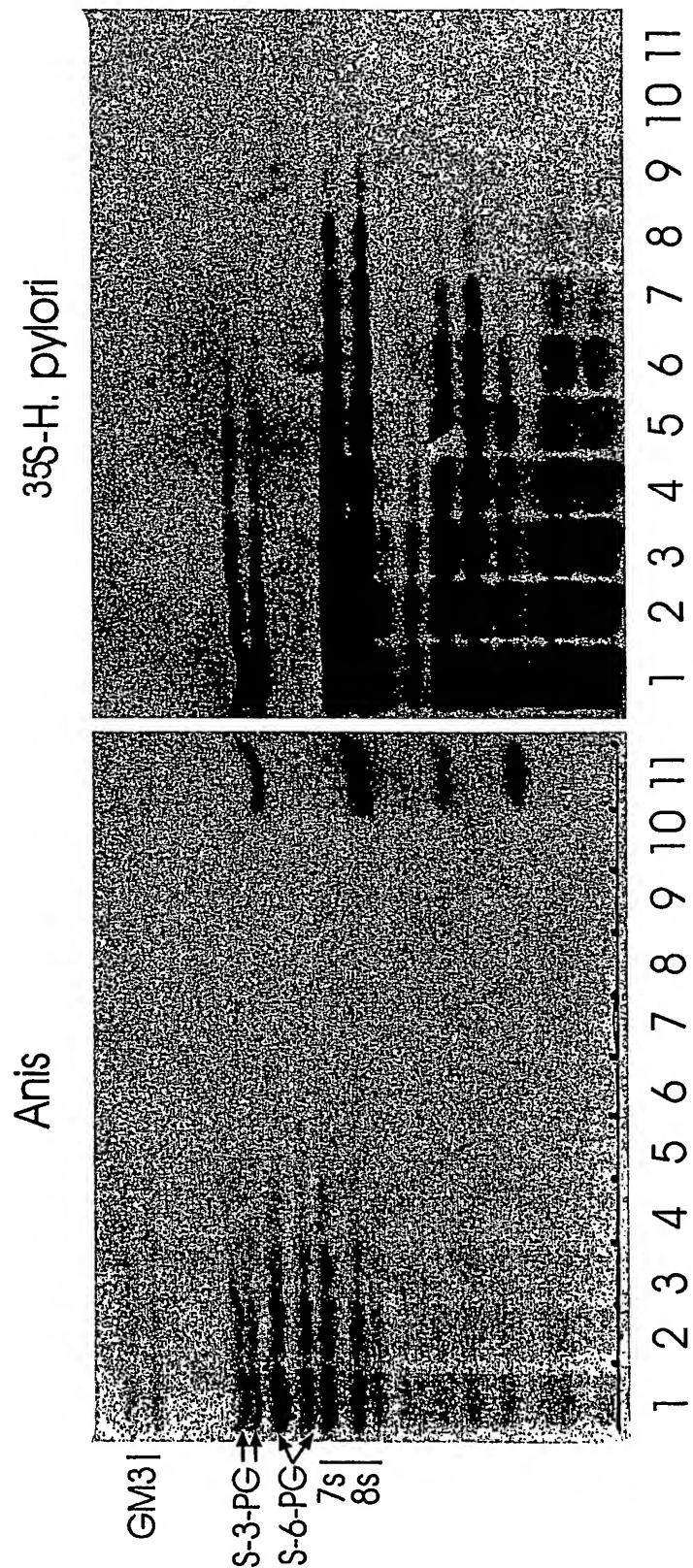


Fig. 11

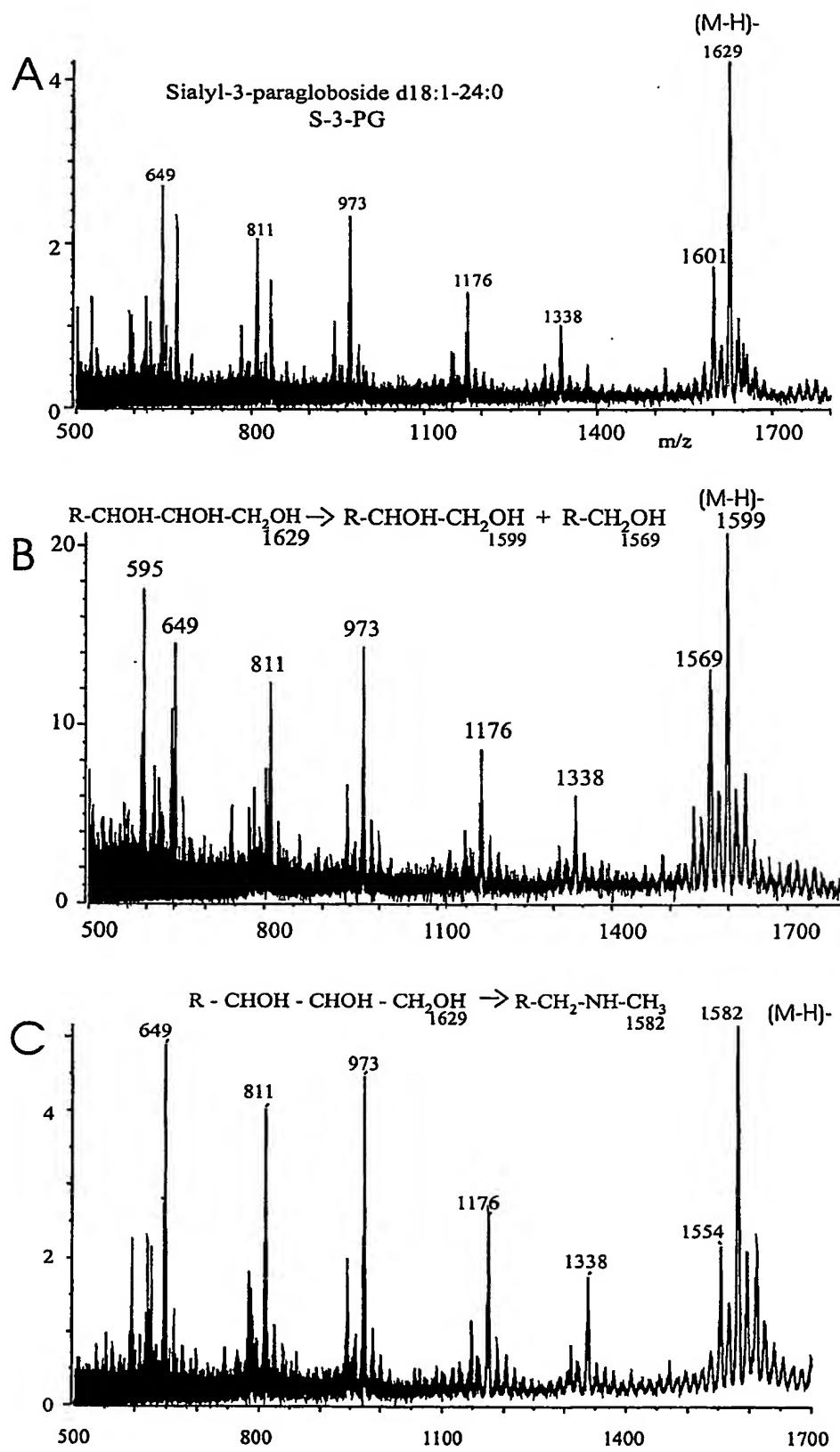
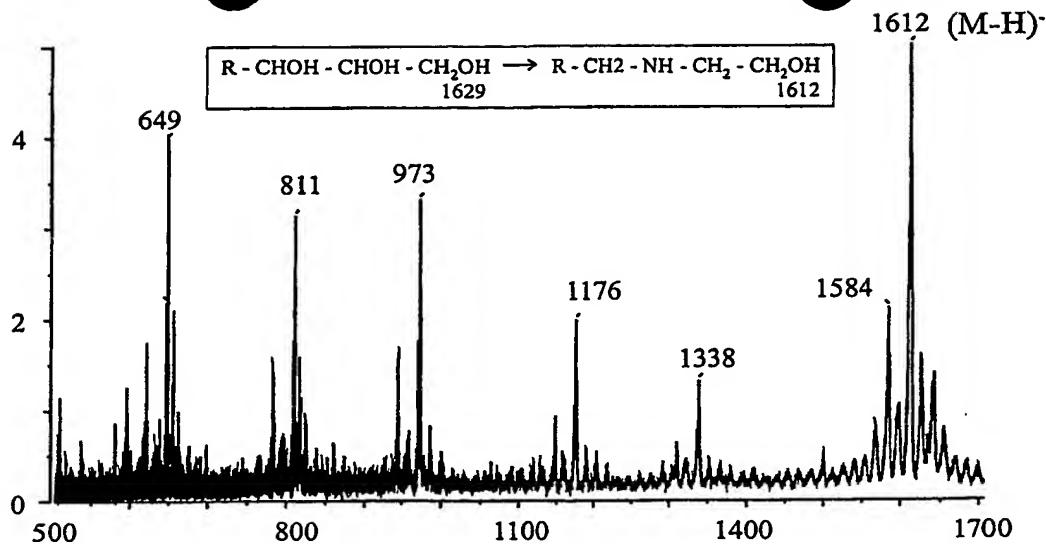


Fig. 12

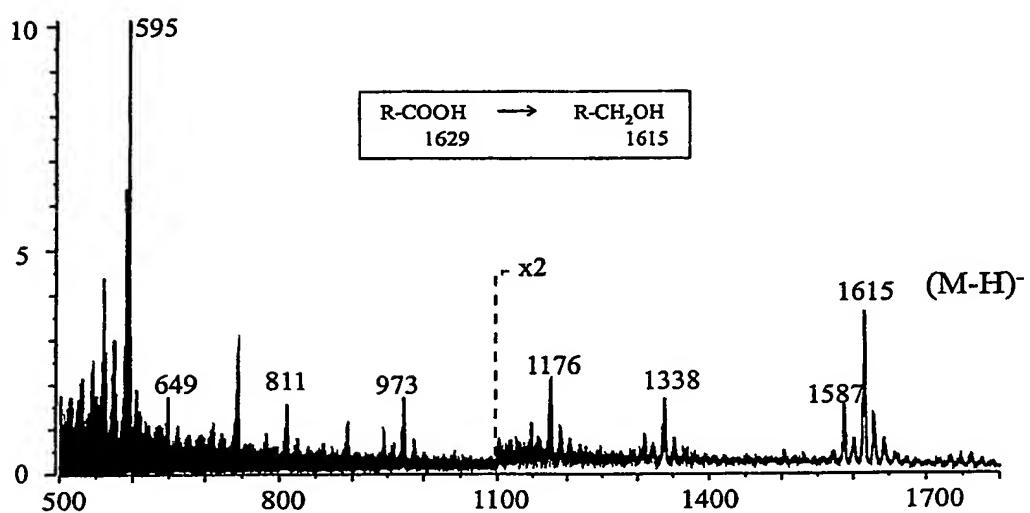
L5

15

D



E



F

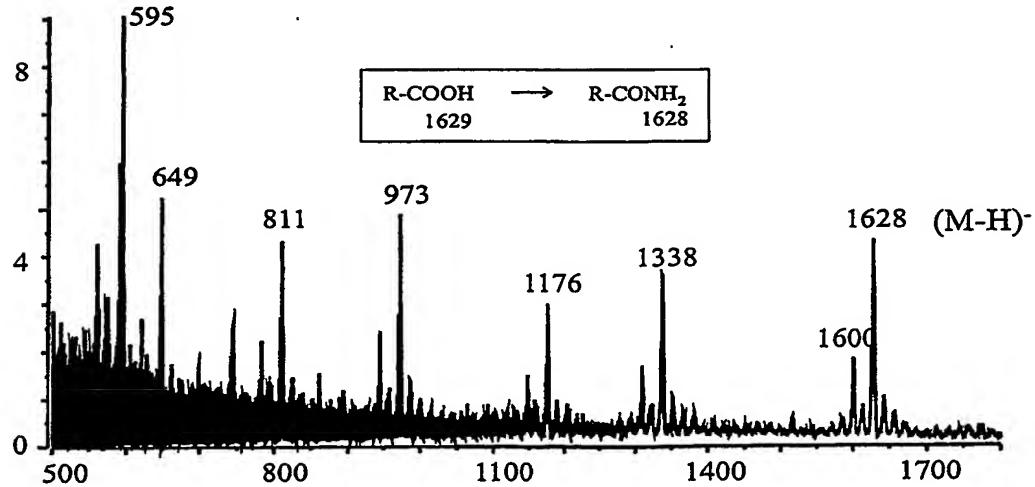


Fig. 12

L5

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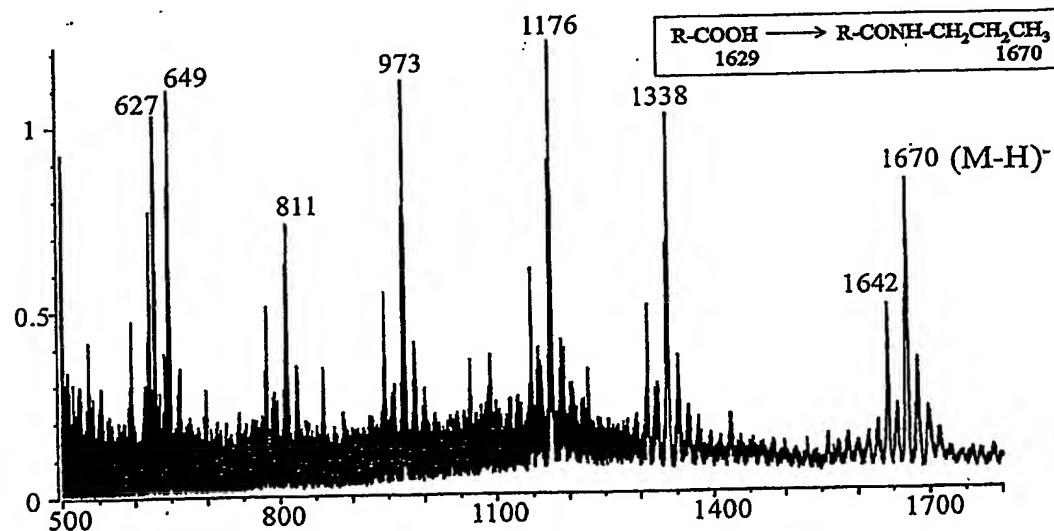
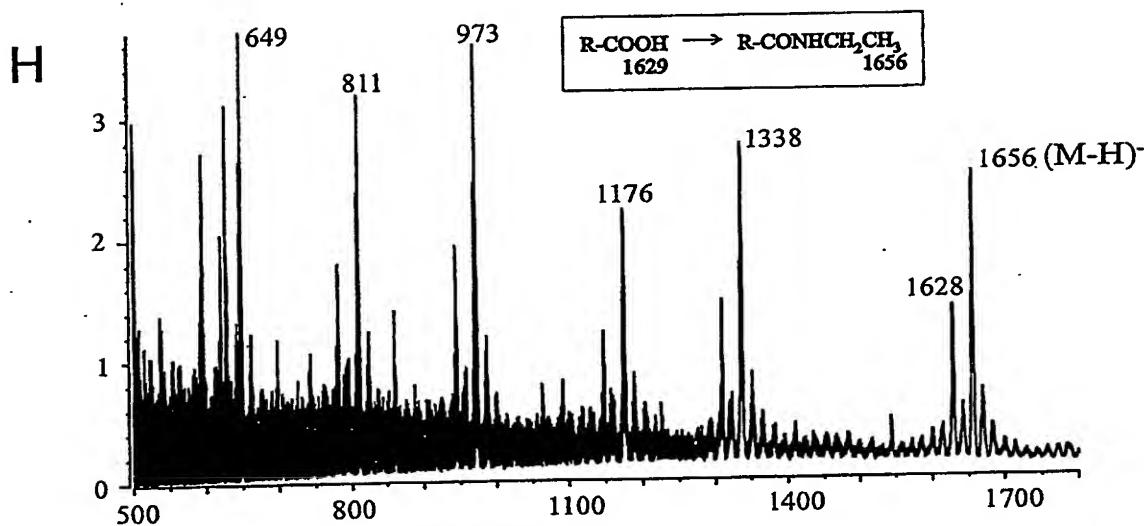
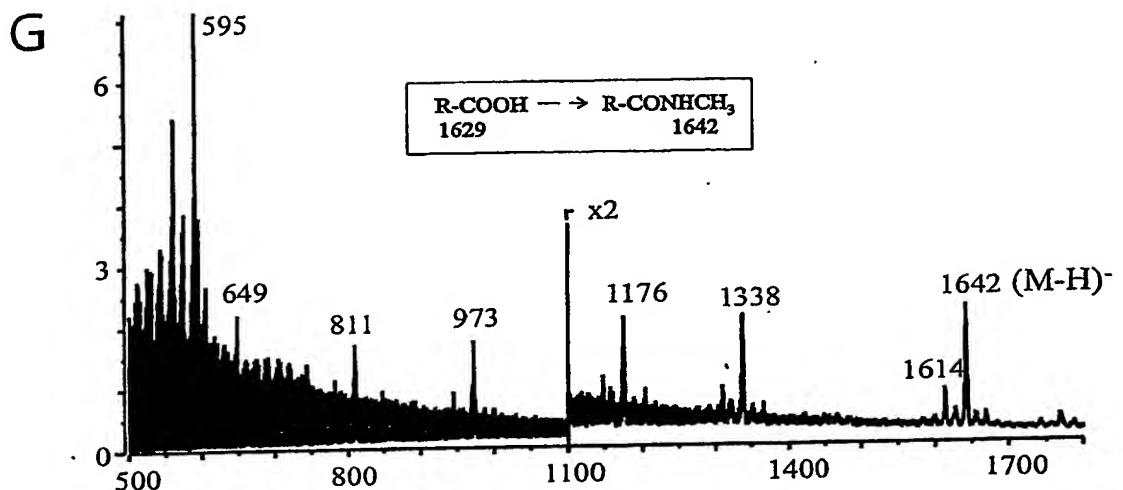


Fig. 12

L5

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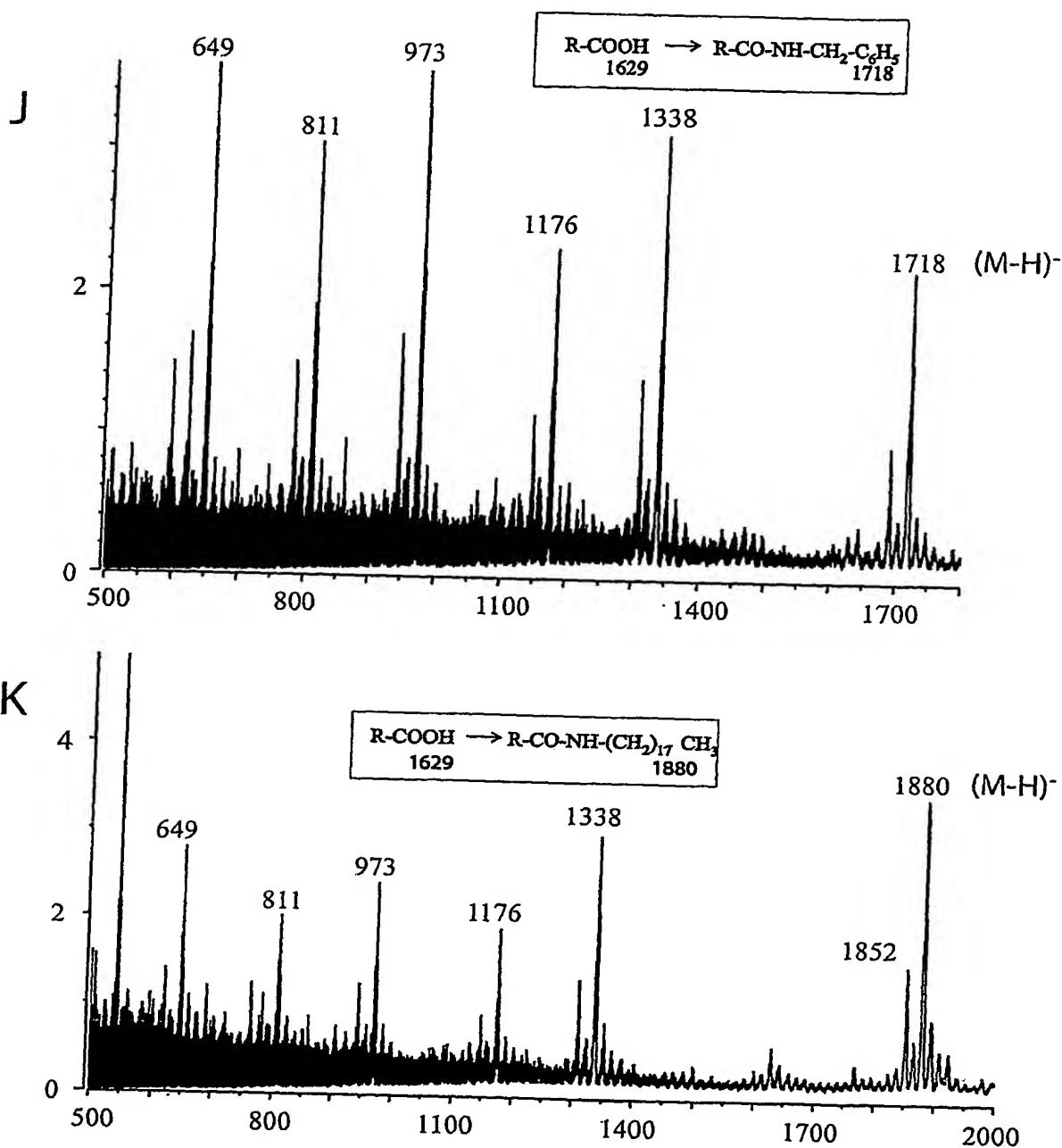


Fig. 12

H. pylori
Anis

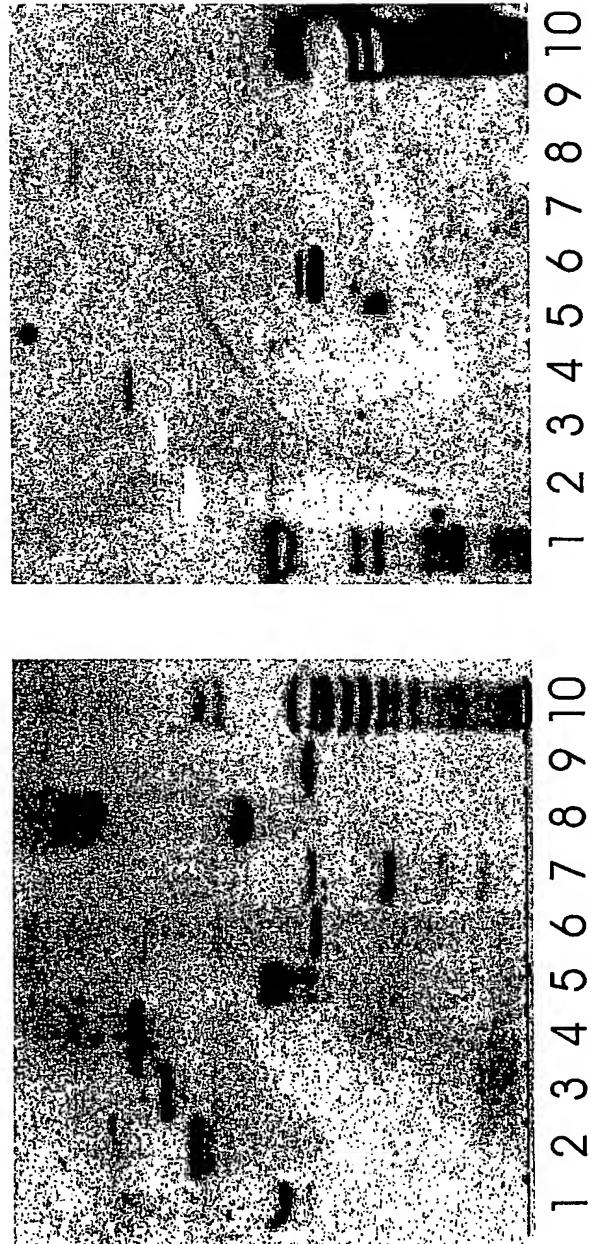
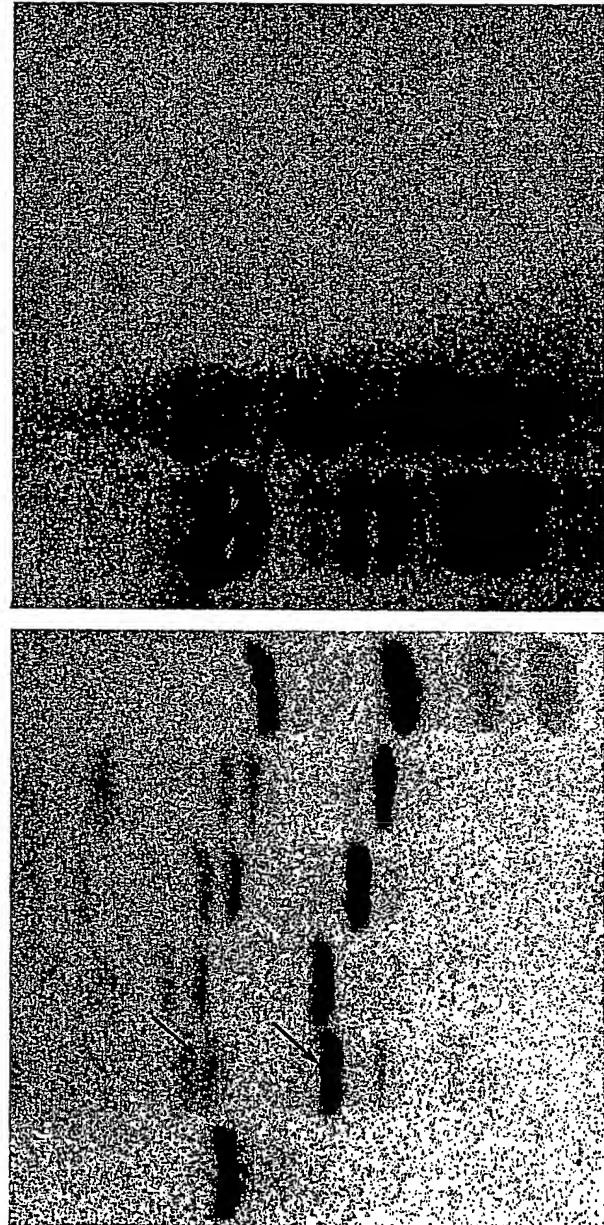


Fig. 13

Anis

H. pylori



1 2 3 4 5 6 1 2 3 4 5 6

L5

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Fig. 14

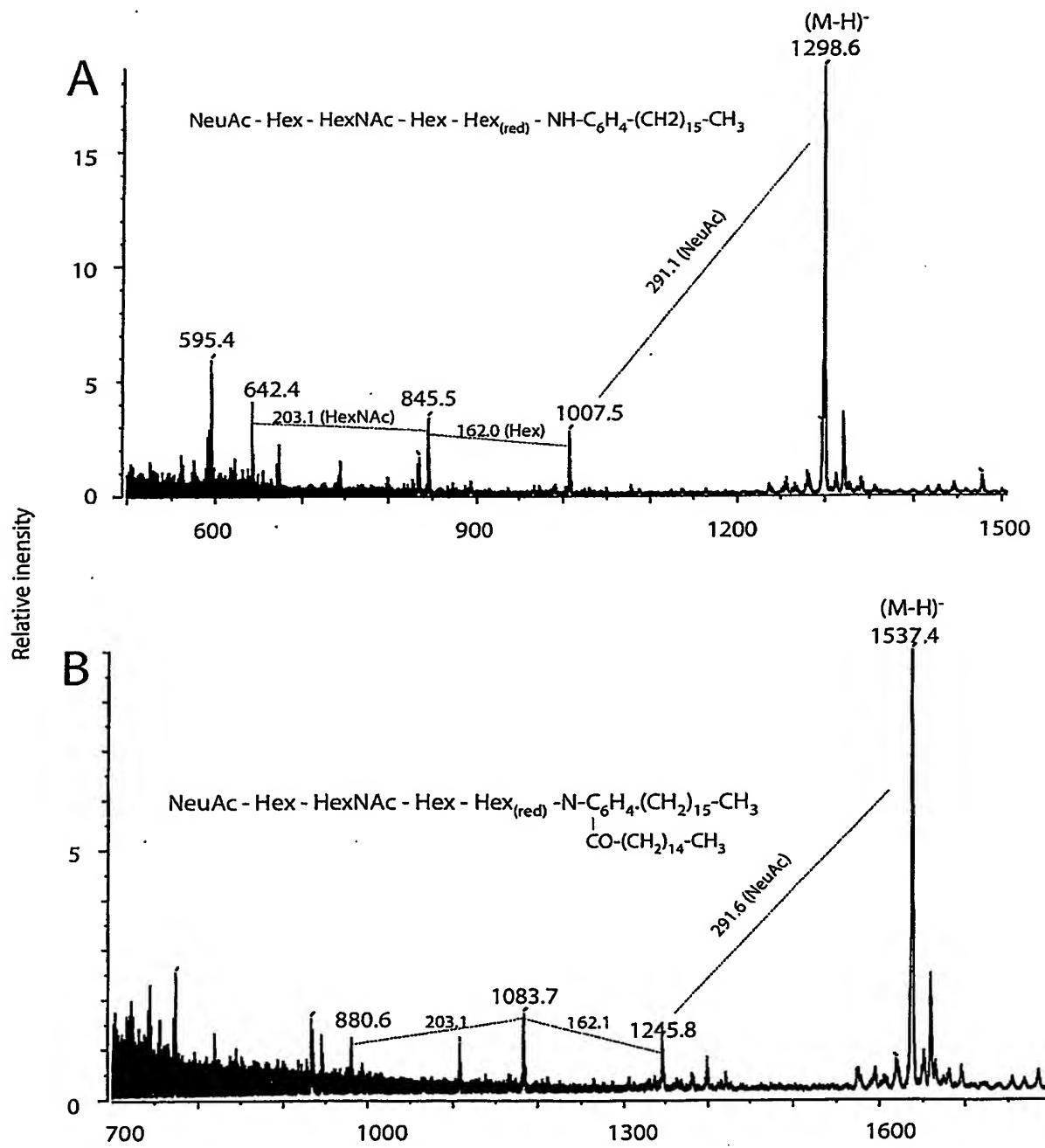


Fig. 15

L 5

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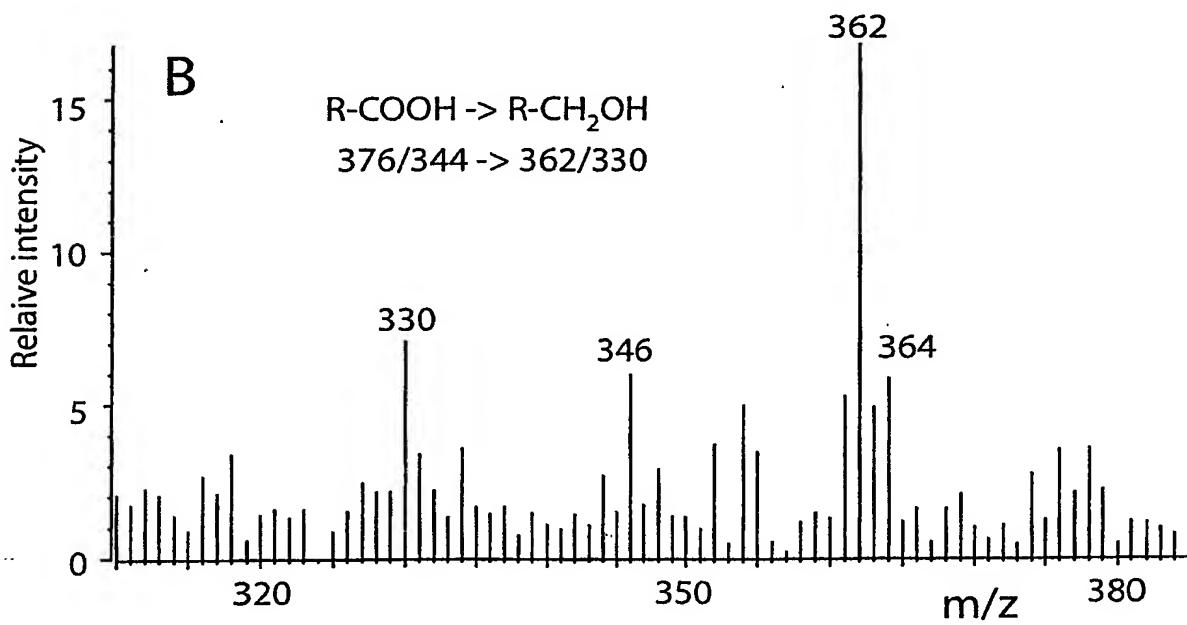
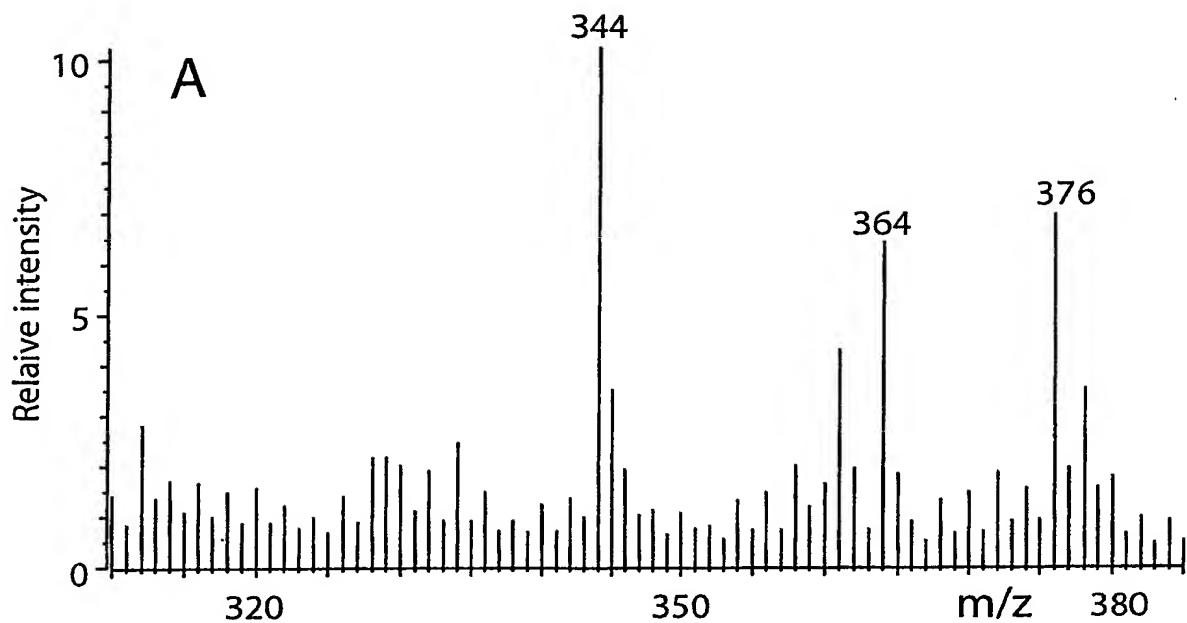


Fig. 16

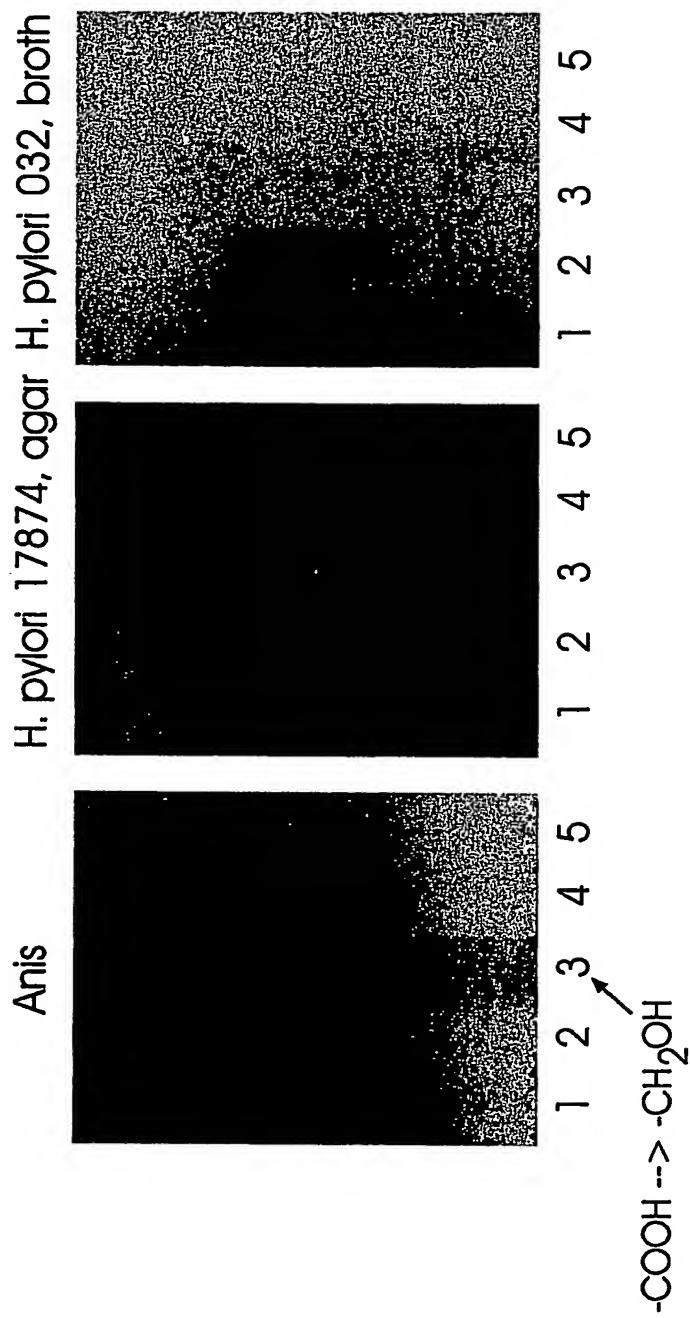


Fig. 17